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ADHERENCE INHIBITION OF *CRONOBACTER SAKAZAKII* AND OTHER PATHOGENS BY PREBIOTIC OLIGOSACCHARIDES, PLANT EXTRACTS, AND OTHER NATURALLY DERIVED MOLECULES

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ADHERENCE INHIBITION OF *CRONOBACTER SAKAZAKII* AND OTHER
PATHOGENS BY PREBIOTIC OLIGOSACCHARIDES, PLANT EXTRACTS,
AND OTHER NATURALLY DERIVED MOLECULES

by

Maria Isabel Quintero V.

A THESIS

Presented to the Faculty of

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Under the Supervision of Professor Robert W. Hutkins

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ADHERENCE INHIBITION OF *Cronobacter sakazakii* AND OTHER
PATHOGENS BY PREBIOTIC OLIGOSACCHARIDES, PLANT EXTRACTS,
AND OTHER NATURALLY DERIVED MOLECULES

Maria Isabel Quintero, M.S.

University of Nebraska, 2011

Advisor: Robert W. Hutkins

For most bacterial pathogens, adherence of the bacterium to the surface of the host cell tissue is a necessary first step for colonization and infection. Agents that inhibit adherence, therefore, could be useful for preventing infections. The goal of this research was to assess the anti-adherent activity of several food-grade prebiotic carbohydrates, plant extracts, and other naturally-derived molecules against enteric pathogens. First, the antiadherent effect of galactooligosaccharides (GOS), polydextrose (PDX), and a GOS-PDX blend was tested against two strains of *Cronobacter* "*Enterobacter*" *sakazakii*. When measured microscopically or by cultural methods, significant reductions in adherence (56% and 71%, respectively) of *C. sakazakii* were observed in the presence of GOS (16 mg/ml). Adherence inhibition also occurred (48%) when the GOS-PDX blend (8 mg/ml each) was tested, although PDX by itself had less

effect. Subsequently, the ability of several prebiotic agents, including chitooligosaccharides (COS) and mannan oligosaccharides derived from yeast cell walls (MOSy) and konjac root (MOSk), to inhibit adherence of enteropathogenic *Escherichia coli* (EPEC), enterohemorrhagic *Escherichia coli* (EHEC) and *Salmonella typhimurium* to a human HEp-2 cell line was tested. In addition, a high molecular weight component of cranberry (CHMW) was also assessed for anti-adherence. Different fractions of COS significantly reduced adherence of EPEC at a concentration of 16 mg/ml. Although MOSy inhibited EPEC, EHEC, and *S. typhimurium*, adherence inhibition was not observed for MOSk. Adherence inhibition of EPEC, EHEC, and *S. typhimurium* by CHMW was observed. Finally, the ability of two different types of lactoferrin to inhibit adherence of *Cronobacter sakazakii* to a HEp-2 human cell line was assessed. Results showed that the adherence of *C. sakazakii* was significantly reduced at a minimum concentration of 10 mg/ml. However, at higher concentrations (up to 50 mg/ml), further reductions in adherence were not observed. These results show that different prebiotics, plant extracts, and other molecules may be added to foods as a prophylactic treatment to prevent or mitigate infections by enteric pathogens.

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“Knowledge is food for the soul” – Plato.

During the past two years I have been immersed in a world which I never imagined it even existed; and I have encountered the most challenging but fascinating experiences in my career of life so far. Graduate school has given me different perspectives of my life and has led me to the understanding of everyday experiences which are normally taken for granted.

I want to give my most sincere thanks to my advisor Dr. Robert Hutkins for his guidance and support during the process of obtaining my degree, as well as to Dr. John Rupnow for his unconditional support and guidance throughout the past two years. Many thanks to my co-workers for all their help with the development and understanding of my research, especially Maria Ximena Maldonado, Maria Elisa Perez, Roberto Jimenez, Lauren Davis, Lyn Oh, Ines Martinez, Richard and Geri Spinner.

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Preface

This thesis is comprised of five chapters. Chapter 1 provides a review of the current literature on the anti-adherent effect of different prebiotic oligosaccharides and plant extracts. Chapter 2 describes our published (Quintero et al. 2011 Current Microbiology) results focusing on the antiadhesive effect of galactooligosaccharides (GOS) and polydextrose (PDX), alone and in combination, against *Cronobacter sakazakii*. Chapter 3 describes the results obtained when testing the antiadherent effect of different prebiotic oligosaccharides and plant extracts against EPEC, EHEC, and *Salmonella typhimurium*. Chapter 4 describes results on the antiadherent effect of lactoferrin against *Cronobacter sakazakii* at different doses. Finally, Chapter 5 provides a conclusion section that summarizes the major research findings presented within this thesis.

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Chapter 1

Prebiotic Oligosaccharides, Plant Extracts, and Naturally Derived Molecules: A review on Their Beneficial Effects and Anti-adherent Activity Against Pathogens

Introduction

Prebiotics are a group of dietary components that have received considerable attention due to their ability to modulate the gastrointestinal microbiota and confer health benefits to the host. Prebiotics were initially defined in rather narrow terms as “non-digestible food ingredients that provide a beneficial effect to the host by stimulating the growth of selected members of the gastrointestinal tract, and thus improve host health” (Gibson et al. 1995). More recently, prebiotics were redefined as “selectively fermented ingredient(s) that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora, that confers benefits upon host well-being and health (Roberfroid 2007). However, by either definition, food ingredients have been considered as prebiotics based exclusively on their ability to cause changes in the intestinal microbiota.

Among the food components considered as prebiotics, most are comprised of soluble oligosaccharides and polysaccharides. Some dietary fibers have also been suggested as having prebiotic characteristics, although not all of them fulfill the requirements to be classified as prebiotic (Roberfroid 2007). To avoid claims that are not fully supported, Roberfroid suggested that a food ingredient can be classified as a prebiotic if: i) it is not hydrolyzed, nor absorbed in the upper part of the gastrointestinal tract, ii) it is a selective substrate for beneficial members of the gastrointestinal tract, which will be stimulated to grow or be metabolically activated, iii) it is able to alter the intestinal microflora in favor of a healthier composition, and iv) it is able to induce systemic effects that are

beneficial to host health (Gibson et al. 1995; Roberfroid 1993; Roberfroid 1998). Non-digestible oligosaccharides such as inulin, galactooligosaccharides (GOS), and fructooligosaccharides (FOS) are now well established as prebiotics; however, more research is needed to determine whether xylooligosaccharides, soybean oligosaccharides, and glucooligosaccharides, should be regarded as prebiotics (Roberfroid 2007; Gibson et al. 2004; Roberfroid 2000).

Despite the criteria described above, studies have been reported in recent years that indicate prebiotics may enhance host health not only by shifting the gut microbiota but by an entirely different biological function. Specifically, several research groups have shown that prebiotics may directly prevent infections by inhibiting the ability of pathogenic bacteria to adhere to target tissues in the host (Shoaf et al. 2006; Gibson et al. 2005; Saulnier et al. 2009). In this chapter, the bifidogenic health benefits of prebiotics will be briefly reviewed, but particular attention will be devoted to their antiadherence activities and the proposed model by which prebiotics can be used to inhibit foodborne infections. Additionally, the antiadherent activity of novel prebiotic oligosaccharides and plant extracts will be addressed.

Prebiotics, oligosaccharides, and human health

Among the most well studied prebiotics are inulin, FOS, and GOS. They are either extracted from natural sources (e.g., inulin from chicory root), produced via hydrolysis of plant polysaccharides (e.g., FOS from inulin) or synthesized enzymatically from sugars found in milk or plant material (e.g., FOS and GOS

from sucrose and lactose, respectively) via glycosyl transferase reactions (Grizard & Barthomeuf 1999; L'Hocine 2000). Interestingly, human milk and colostrum from lactating animals also contain high concentrations (> 1%) of oligosaccharides (Coppa et al. 2008) that have prebiotic activity.

That these two groups of oligosaccharides (i.e., those that are food grade and those are obtained from human milk) have general structural similarities has led to the suggestion that they may also share similar biological activities. It is well known that human milk is already a very rich source of nutrients, including oligosaccharides that are important for the health of the infant during the first few months of life (Coppa et al. 2006). This oligosaccharides fraction, which can reach concentrations as high as 8 to 12 g/L (Kunz et al. 2000), is comprised of sialic acid, N-acetylglucosamine, L-fucose, D-glucose, and D-galactose. Galactose residues form three different types of oligosaccharides found in human milk: 3'-galactosyl-lactose, 4'-galactosyl-lactose, and 6'-galactosyl-lactose (Boehm et al. 2005; Torres et al. 2010). The absence of fucose and amine groups in commercial GOS products distinguishes these OS from human milk OS and has led some researchers to argue that these structural differences "do not support a direct parallel functionality and thus, need to be further investigated" (Kunz et al. 2009).

The effect of prebiotics on the human gastrointestinal microbiota has been the subject of extensive study. This interest has been due largely to the bifidogenic properties of prebiotics and the many observations showing that high levels of bifidobacteria in the intestinal tract are correlated with health benefits in

humans. Specifically, these organisms have been associated with increased resistance to infection and diarrhea (Saavedra 1994; Tojo et al. 1987; Yamazaki et al. 1985), stimulation of the immune system (Kirjavainen et al. 2002; Sekine et al. 1985), and protection against cancer (Reddy et al. 1993) as well as increased production of short chain fatty acids. Convincing evidence has also emerged showing that bifidobacteria are the main species colonizing the infant gastrointestinal tract and that a bifidobacteria-rich microbiota protects the infant against pathogenic bacteria (Stark et al. 1982; Moro et al. 2002; Penders et al. 2006; Weizman et al. 2005). However, the proportion of bifidobacteria decreases with age, thus efforts to restore or increase this population are warranted.

Galactooligosaccharides (GOS)

Commercial GOS is enzymatically synthesized from lactose using glycoside hydrolases (Roode et al. 2003) that catalyze transgalactosylation reactions (Crittenden et al. 1996). Resulting products have different linkages depending on the enzyme source. Commercially available GOS commonly includes β -1,3, β -1,4, or β -1,6 linkages (Torres et al. 2010). Like other prebiotics, GOS selectively stimulates the growth of bifidobacteria and lactobacilli in the host gastrointestinal tract (Teuri et al. 1998; Moro et al. 2002; Bouhnik et al. 2004; Depeint et al. 2008). Doses necessary to elicit a bifidogenic response vary from 2.5 to 10 g per day (Moro et al. 2002; Sako 1999; Depeint et al. 2008; Silk et al. 2009). However, recent studies also suggest that the bifidogenic effect of GOS is not only dose dependant, but relies on undefined host factors (Davis et al.

2010). GOS is marketed throughout the world and is self-affirmed as GRAS in the U.S.

Inulin

Inulin, a naturally-occurring plant polysaccharide, is one of the most widely consumed prebiotics. It is a β -2,1 fructan with a degree of polymerization (DP) ranging from 2 to 60 (Costabile et al. 2010). Inulin is characterized for having a bifidogenic effect when consumed in a regular basis. Human trials have been reported that show significant increases in bifidobacteria after regular consumption of inulin at doses ranging from 5 to 40 g/day (Kolida et al. 2007; Kruse et al. 1999; Gibson 1995; Kleessen et al. 1997; Kleessen et al. 2007; de Preter et al. 2008). Inulin is legally classified as a food ingredient in all the countries in which it is used .

Fructooligosaccharides (FOS)

Two forms of fructooligosaccharides (FOS) are produced commercially. One form is derived from hydrolysis of inulin and consists mainly of β -2,1 fructose oligosaccharides, along with fructooligosaccharides chains containing glucose at the terminal end. The other commercial form of FOS is obtained enzymatically from sucrose via a transfructosylation reaction. This FOS contains a mixture of oligosaccharides, each consisting of glucose linked to two, three, or four fructose units (Molis et al. 1996). Human trials where FOS was consumed on a regular basis showed an increase in bifidobacterial levels; daily doses ranged from 2.5 to

20 g/day. (Bouhnik et al. 1999; Tuohy et al. 2001; Buddington et al. 1996; Menne et al. 2000; Bouhnik et al. 2007; Guigoz 2002; Williams et al. 1994)

Xylooligosaccharides (XOS)

Xylooligosaccharides are sugar oligomers made up of xylose units. They are found naturally in bamboo, fruits, vegetables, milk, and honey and are industrially produced from lignocellulosic materials (LCM) which come from a variety of feedstocks (Vázquez 2000). There are three different approaches used to produce XOS from xylan-rich feedstocks: a) enzyme treatments of native, xylan containing LCM, b) chemical fractionation of LCM to isolate or solubilize xylan, with further enzymatic hydrolysis of the polymer to XOS, and c) hydrolytic degradation of xylan to XOS by steam, water or dilute solutions of mineral acids (Vázquez 2000). As for other prebiotics, XOS have been reported to modulate the gut microbiota, especially stimulating the growth of *Bifidobacteria* (Rycroft et al. 2001; Zampa et al. 2004). Although data on human studies is limited, studies have shown increases of 10 to 30% in bifidobacteria following consumption of XOS.

Polydextrose (PDX)

Polydextrose (PDX) is a polysaccharide synthesized by random polymerization of glucose and sorbitol, using an appropriate acid catalyst at high temperature and partial vacuum conditions. It is widely used as a low calorie bulking agent in prepared foods (Jie et al. 2000). Unlike other prebiotics, PDX is only partially fermented by intestinal microorganisms, due to the nature of the

glycosidic bonds, and high amounts are excreted in feces (Achour et al. 1994; Figdor et al. 1981). The partial fermentation in the large intestine leads to reduced transit time, softer stools, and lower fecal pH (Achour et al. 1994). The prebiotic activity of PDX remains unclear, although there are reports that it promotes proliferation of favorable microflora, including bifidobacteria, and decreases bowel pH (Jie et al. 2000).

Mannan oligosaccharides (MOS)

Mannan oligosaccharides (MOS) are derived via partial hydrolysis of the polysaccharide, mannan. The latter consist of α -1,4linked mannose monomers. They are obtained commercially either from plant material or from yeast cell walls. MOS are widely used in cattle, swine, and poultry feed.(Dimitroglou et al. 2009) They have been shown to improve gut function and health by increasing the integrity, uniformity and height of the villi, which results in a higher absorption efficiency in the gastrointestinal tract. (Iji et al. 2001; Hooge 2004; Castillo et al. 2008; Spais et al. 2003; Sims et al. 2004) Supplementation of poultry diets with MOS has shown a positive response in body weight and feed conversion; especially with cage-housed commercial broiler chicks (Kumprecht et al. 1997). In addition, a study by Savage et al. 1996 showed that inclusion of MOS at 1 g/kg diet enhanced both IgG and IgA serum antibody levels in turkey poults. The benefits obtained from diet supplementation with MOS are attributed with several factors, including modification of the intestinal microflora of the animals (Shane et al. 2001).

Pectic oligosaccharides (POS)

Pectins are complex polysaccharides that represent one of the major components of the plant cell wall of dicotyledonous plants (Willats 2000; Mandalari et al. 2006). They are considered soluble dietary fiber and exert physiological effects on the gastrointestinal tract consistent with other fibers, in that they delay gastrointestinal emptying (Flourie et al. 1985), reduce gut transit time (Spiller et al. 1980), and reduce glucose absorption rates (Jenkins et al. 1977). Pectic oligosaccharides (POS) are formed via enzymatic hydrolysis of pectins. Pectin is a heteropolysaccharide and contains several sugars, but the primary monomers are α -1,4-linked galactosyluronic acid residues. The enzymatically generated POS have been reported in several *in vitro* studies to have prebiotic activity (Manderson et al. 2005; E. Olano-Martin et al. 2002), based on increases in bifidobacteria.

Chitooligosaccharides (COS)

Chitin is a linear polysaccharide consisting of β -1, 4 linked N-acetyl-D-glucosamine residues. It is considered as one of the most abundant polysaccharides in nature, after cellulose. It is mainly found in the cell walls of fungi and yeasts and the exoskeleton of arthropods and insects (Aam et al. 2010; Minke et al. 1978). Partial deacetylation of chitin yields chitosan, a heteropolymer of N-acetyl-D-glucosamine and D-glucosamine residues. Chitooligosaccharides (COS) are oligomers that can be obtained from chitosan either chemically or enzymatically via synthesis with glycosyl hydrolases (Aam et

al. 2010). Although the prebiotic activity of COS remains unclear, reports have indicated that COS has antimicrobial activity mediated, in part, by adherence inhibition (Rhoades et al. 2006; Xia et al. 2011).

The antiadherence model

Attachment of pathogenic bacteria to host cell surfaces is the first and in many cases, one of the most important steps that occur during the infection process (Klemm et al. 2010; Bavington et al. 2005; Shoaf et al. 2006). Bacterial adhesion is mediated by several different physical and biological processes, as shown in Figure 1. In general, physical forces influence the proximity of the bacteria to the surface of the host cell only up to about 5 nm (Busscher 1987). A much stronger and mostly irreversible complex is then formed between specific bacterial adherence proteins and complementary host receptors (Pinzón-Arango et al. 2009; Abu-Lail et al. 2003). These specific interactions are mediated primarily by lectin-like molecules that recognize specific carbohydrates located at the surface of epithelial cells. Lectins are defined as structurally diverse, carbohydrate-binding proteins. Bacterial lectins are organized as thin-thread-like organelles called fimbriae or pili (Bavington et al. 2005; Klemm et al. 2010; Sharon 2006). The structural differences between different lectins are based on a small globular carbohydrate-recognition domain; slight chemical differences in these domains allow for the selectivity of each adhesin to its target receptor (Shoaf-Sweeney et al. 2008; Weis et al. 1996). Thus, the specificity of a pathogen for a particular tissue or host is dependent on the type of oligosaccharide receptors coating the epithelial cell surface (Shoaf-Sweeney et

al. 2008). Bacterial attachment not only enables the organism to avoid displacement by the host's natural cleansing mechanisms (e.g., peristalsis, bile acid excretion, flux, etc.), but also provides access of the organism to sources of nutrients located at the epithelial cell surface. Ultimately, bacterial adherence promotes the organism's potential for colonization and infection (Sinclair et al. 2009). Bacteria that are incapable of expressing functional adhesins are not able to adhere and initiate infections, suggesting that adherence for these bacteria is an essential step in pathogenesis (Boddicker et al. 2002; Cleary 2004). Importantly, these observations imply that agents that block the initial attachment of potential pathogens to host cell surfaces could significantly reduce or prevent bacterial infections (Ziegler et al. 2007).

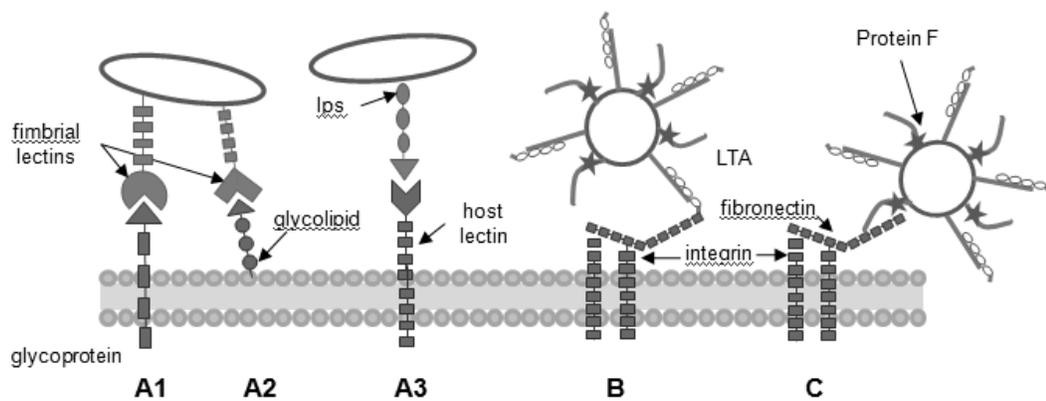


Figure 1. Bacterial adhesion involving lectin-carbohydrate interactions: bacterial lectin-host glycoprotein (A1); bacterial lectin-host glycolipid (A2); host lectin-bacterial LPS (A3); hydrophobin-protein interactions (B); protein-protein interactions (C). Adapted from Ofek et al. 2003.

One group of substances that have been considered as anti-adherence agents includes prebiotic oligosaccharides. Accordingly, they are proposed to act as molecular decoys that mimic the carbohydrate binding sites recognized by the pathogen. The model is based on the observation that certain prebiotic oligosaccharides have similar structures to the ones found in the surface of epithelial cells (Kunz et al. 2000; Shoaf et al. 2006). Once inside the GI tract, the pathogen will recognize the prebiotic as a binding site, adhere, and be removed from the system. Oligosaccharides that have been shown to have anti-adherence activity include oligosaccharides derived from human milk, as well as the commercial prebiotics, GOS, FOS, inulin, lactulose, and raffinose (Shoaf et al. 2006). Plant extracts and other molecules have also been tested and reported to have anti-adherence activity. Several of these will be briefly reviewed in the next section.

Anti-adherent oligosaccharides from human milk

Human milk is not only a rich source of protein, fat, and other nutrients, but it also contains a large carbohydrate fraction (7%) consisting of lactose and a complex mixture of oligosaccharides (Zivkovic et al. 2010); between 5 and 23 g/L. (Kunz et al. 2000; Coppa et al. 1993). Many of these human milk oligosaccharides (HMO) have similar structures to those carbohydrates found in the surface of intestinal epithelial cells (Sharon et al. 2000; 2002). More than 130 different HMO's (Kunz et al. 2000), are known to be produced during the first

several months of lactation (Ben et al. 2004) More recent analyses have identified as many as 200 molecular species in pooled human milk samples (Zivkovic et al. 2010). The basic structure of HMOs is comprised of lactose at the reducing end, extended by N-acetyllactosamine units. The structural diversity is granted by fucose and sialic acid residues at the terminal positions (Zivkovic et al. 2010). The structures of the identified HMO's consist of neutral and acidic oligosaccharides, terminated by fucose (50 – 70%) and sialic acid (5 -15%). (Ninonuevo et al. 2006) When tested *in vitro*, HMO have been reported to have high affinity for bacterial adhesins (Newburg 2000; Miller et al. 1994; Newburg et al. 2004; Kobata 2003). They have been reported to inhibit adhesion of *Escherichia coli*, *Vibrio cholerae*, and *Salmonella fytis* to a Caco-2 tissue culture cell line (Coppa et al. 2006). Other studies have reported that HMO inhibit adherence of *Campylobacter jejuni* (Ruiz-Palacios et al. 2003), *Streptococcus pneumoniae* and *Haemophilus influenzae* to human cell lines (Andersson et al. 1986). Despite their effectiveness as antiadhesives agents, they are obviously are not food grade and cannot be used as food ingredients.

Galactooligosaccharides as anti-adherent agents

Commercially available GOS has been tested for antiadherent activity against several enteric pathogens. Studies have shown that GOS inhibits the adherence of *Escherichia coli*, *Salmonella enterica* serovar *Typhimurium* and two different strains of *Cronobacter sakazakii* to tissue culture cells at a concentration of 16mg/ml (Shoaf et al. 2006; Quintero et al. 2011; McGowan et al. 2011 manuscript in preparation) and suggested that anti-adherent activity against *E.*

coli seems to be dose dependant until it reaches a plateau at a concentration of 16 mg/ml. Searle et al. also reported adherence inhibition of *S. typhimurium* by GOS in a mouse model (Searle et al. 2009).

Mannan oligosaccharides as anti-adherent agents

It is well known that the Type 1 fimbriae from *Salmonella* sp. and *Escherichia coli* are mannose-sensitive, in that free mannose is capable of inhibiting adherence of these bacteria, as well as other pathogens, to host tissues (Firon 1982; Neeser et al. 1986; Old 1972; Salit 1977; Kisiela et al. 2006) Ganan et al. 2009 reported that yeast derived mannoproteins inhibit adherence of *Campylobacter jejuni* to a Caco-2 cell line. Their results indicated that the availability of mannose in the mannoprotein fraction seemed to be highly important for inhibiting adherence and invasion. Furthermore, protective effects with dietary mannose were also reported against *Campylobacter jejuni* colonization in chickens (Schoeni et al. 1994). Other studies have shown that adherence of *Escherichia coli* to epithelial cells can be inhibited or reversed by the addition of mannose and its derivatives. (Ofek et al. 1978) Moreover, α -D-mannopyranoside has been shown to prevent urinary tract infection in mice by inhibiting adherence and further colonization of the urinary tract by *E. coli* (Aronson et al. 1979). Another study showed that chicks challenged with *Salmonella typhimurium* had decreased cecal colonization when mannan oligosaccharides were added to their diet (Spring et al. 2000).

Pectic oligosaccharides as anti-adherent agents

Pectic oligosaccharides have been reported to inhibit invasion of *Campylobacter jejuni* to Caco-2 cells in a dose dependent manner. (Ganan et al. 2010). Olano-Martin et al. 2003 assessed the ability of pectic oligosaccharides to neutralize *Escherichia coli* Shiga toxins at various doses. A concentration of 10 mg/ml was reported to completely inhibit Shiga toxin 1 and Shiga toxin 2. Despite these results, however, further research is needed to determine antiadherence properties against other pathogens.

Other anti-adherent agents

In addition to oligosaccharides, some plant extracts and glycoproteins have been reported to have antiadherence activity. Some of the mechanisms by which they inhibit bacterial adherence have been well studied, while others need further research. Of particular interest is the large molecular weight fraction from cranberry concentrate and lactoferrin, a glycoprotein found in high concentrations in milk.

Anti-adherent properties of cranberry

Cranberry juice has been consumed for decades for the purpose of treating and preventing urinary tract infections. Urinary tract infections (UTI) are one of the most prevalent diseases among females; approximately eight million people per year experience UTIs in the U.S. (Cohn et al. 2004). They are most often caused by Gram-negative bacteria, primarily uropathogenic *Escherichia coli* (UPEC) (Johnson 2003). Although UTIs can usually be treated successfully with antibiotics, when infections become recurrent, there is a growing concern about

antibiotic resistance (Liu et al. 2006; Wilson et al. 2004). Recently, oral consumption of cranberry products was reported to reduce UTI's (Raz et al. 2004). The ability of cranberry to reduce or prevent infections caused by UPEC has been attributed to the presence of two different compounds in cranberry products; a high molecular weight component and proanthocyanidins (PACs). Both were reported to inhibit adhesion of UPEC to uroepithelial cells (Rahbar et al. 2010). Zafriri et al. 1989 reported that the non-dialyzable material of a cranberry cocktail inhibited the activity of a mannose-specific Type 1 fimbriated *E. coli*. This was assayed by yeast aggregometry, hemagglutination, adherence to tissue culture cells, and attachment to mouse peritoneal macrophages. The mechanism by which cranberry inhibits adhesion is poorly understood, although it has been reported that some components interact directly with P-fimbriae by altering their conformation and by binding of hydrophilic components, hence inhibiting adherence (Liu et al. 2006). Interestingly, Sobota 1984 reported that urine from mice and human still contained components that reduced *E. coli* adherence after the consumption of cranberry products, suggesting that the active compounds might not be destroyed during digestion. Other studies, both *in vitro* and *in vivo*, have shown that cranberry juice constituents inhibit adhesion of *Helicobacter pylori* (Burger et al. 2000; Burger et al. 2002; Shmueli et al. 2004). Furthermore, the high molecular weight component in cranberry juice has been shown to reduce salivary *Streptococcus mutans* in a randomized controlled population (Weiss et al. 2004). The effect was attributed to the ability of the

HMW component to inhibit adhesion and biofilm formation of *Streptococcus* onto tooth surfaces. (Steinberg et al. 2005; 2004; Weiss et al. 2004)

Anti-adherent properties of lactoferrin

Lactoferrin is an iron-binding protein that is found in high concentrations in human milk and in lower concentrations in other fluids (Legrand et al. 2005). It can be found in human colostrum in concentrations up to 7g/l, (Masson et al. 1971) and up to 1 mg/ml in human milk after the first month of lactation. In bovine colostrum, it can reach up to 1.5 mg/ml. (Theresa J Ochoa et al. 2009). It is a very stable protein and is highly resistant to proteolytic degradation (Lönnerdal 2009). Lactoferrin can be recovered in the stool of breast fed infants, indicating it is at least partially resistant to digestion in the gut (Davidson et al. 1987).

Several biological functions have been attributed to lactoferrin, including antimicrobial, anti-inflammatory, and immunomodulatory activities (Brock 2002; Kruzel et al. 2002; Ward et al. 2002; Legrand et al. 2008; Actor et al. 2009). One of the first mechanisms that account for the antimicrobial properties for lactoferrin was based on its ability to sequester iron, thus depriving pathogens from this essential nutrient (Bullen et al. 1972; Arnold et al. 1980; Nicola Orsi 2004). Although lactoferrin is effective against several pathogens, this activity is markedly reduced upon iron supplementation (Arnold et al. 1980; Kalmar et al. 1988; Yamauchi et al. 1993; Valenti et al. 2005). There are also iron-independent mechanisms by which lactoferrin inhibits pathogens, specifically via the direct interaction of lactoferrin with bacterial cell surfaces (Dalmastri et al.

1988; Carol A. Bortner, Roland R. Arnold 1989). Lactoferrin binds the lipid A component of the lipopolysaccharide (LPS) in Gram-negative bacteria, affecting membrane permeability, (Appelmeik et al. 1994; Brandenburg et al. 2001; Shahriar et al. 2006) and thus releasing LPS from the membrane (Appelmeik et al. 1994; Brandenburg et al. 2001; Ellison et al. 1988).

Several other iron-independent activities of lactoferrin exist that exert an antimicrobial effect. It has been demonstrated that lactoferrin has a serine protease-like activity and it is able to proteolytically degrade two colonization factors expressed by *Haemophilus influenzae*, attenuating its virulence and preventing colonization (Qiu 1998; Jenssen et al. 2009). Lactoferrin may also interfere with the Type III secretory system that is used by many pathogenic bacteria to adhere and infect host cells (Ochoa et al. 2003; DeVinney et al. 1999; Goosney et al. 1999). Studies have shown that lactoferrin is able to degrade virulence proteins secreted by *Shigella flexeneri*, resulting in inhibition of bacterial uptake into host cells (Gomez et al. 2001; Gomez et al. 2003). A similar result was observed with enteropathogenic *Escherichia coli*, where lactoferrin caused loss and degradation of several Type III secretion proteins, resulting in reduced bacterial virulence and adherence to host cells (Ochoa et al. 2003). Additionally, enterotoxigenic *Escherichia coli* (ETEC) strains were reported to bind a significant amount of lactoferrin (Naidu et al. 1991; Giugliano et al. 1995), which may have accounted for the adherence inhibition observed for ETEC on HEp-2 tissue culture cells (Giugliano et al. 1995). Similarly, Bessler et al. 2006 reported

that the direct interaction of lactoferrin with the bacterial surface inhibits adherence and invasion of *Salmonella typhimurium*.

Prebiotics as therapeutic agents against the emerging pathogen

Cronobacter sakazakii

Infections caused by foodborne pathogens continue to be a major concern to the food industry, consumers, and public health authorities. Often these infections are self-limiting, but even for more serious cases, few therapies are available. In general, even antibiotics are often of limited value. Research efforts aimed at identifying new therapeutic or prophylactic strategies are attracting considerable attention. Targets for such approaches include not only pathogens, such as *Escherichia coli* and *Salmonella* spp., that have long been associated with foodborne disease, but also recently recognized pathogens, such as *Cronobacter* spp.

The genus *Cronobacter* is located within the family of the Enterobacteriaceae. They are motile, non-sporeforming, rod shaped, Gram negative facultative anaerobes (Chenu et al. 2009). It now appears that all *Cronobacter* species are opportunistic pathogens (Johler et al. 2010; Hartmann et al. 2010). Some species, in particular, *Cronobacter sakazakii*, are responsible for causing rare, but severe forms of meningitis, sepsis and necrotizing enterocolitis (NEC) in newborn infants and neonates (Acker et al. 2001; Hunter et al. 2008; Muytjens et al. 1983; Biering et al. 1989; Bar-Oz et al. 2007). The infant mortality rate due to infection of this organism has been reported to be 40 – 80%,

and up to 20% of neonates that survive develop serious neurological complications (Iversen et al. 2003). Although there is little information about the natural reservoir of this organism, it has been isolated from food processing plants and food products, including cheese products, cured meats, vegetables, and herbs and spices. However, it is the association of *C. sakazakii* with powdered infant formula (PIF) that has attracted the most concern. Indeed, PIF has been implicated in several outbreaks (Forsythe 2005) and sporadic cases of *C. sakazakii* infections (Iversen et al. 2003). Powdered infant formula is now recognized as one of the main food sources for this organism (Iversen et al. 2004; Weir 2002; Drudy et al. 2006; Mullane et al. 2006; Iversen 2004; Iversen et al. 2003). In addition, the organism has been isolated from powdered milk processing plants as well as hospital utensils (spoons and blenders) used to prepare infant formula (Forsythe 2005).

Several factors contribute to the ability of *C. sakazakii* to persist in dry milk and PIF products. The organism has a remarkable resistance to osmotic stress and desiccation, compared with other members of the Enterobacteriaceae, and can persist under desiccated conditions in infant formula for over two years (Breeuwer et al. 2003; Caubilla-Barron et al. 2007). Additionally, it produces a yellow pigment that may protect the cells against UV rays and also has the ability to form capsules and fimbriae that enable the organism to adhere to different surfaces (Mullane et al. 2006). These physiological characteristics not only enhance survival in the food production environment, but may also contribute to persistence in other environmental sources such as water, soil, and vegetables.

Although the incidence of *Cronobacter* infections is unknown, CDC has estimated infection rates as low as 3 cases per year, worldwide. However, because *Cronobacter* infections are most likely to involve newborn infants and neonates, with mortalities rates as high as 40%, the organism is still considered a serious public health threat. The infectious dose has not been established although the Food and Agriculture Organization / World Health Organization (2004) has determined a tentatively oral infectious dose that ranges from 10^3 CFU to 10^8 CFU.

The means by which *C. sakazakii* causes disease has only recently been investigated. The organism appears to be able to trespass the intestinal barrier and establish a systemic infection (Kim et al. 2008). Kothary et al. 2007 determined that *Cronobacter* synthesizes a zinc-containing metalloprotease that has collagenolytic activity that allows the organism to cross the blood-brain barrier. Hunter et al. 2008 suggested an association of *Cronobacter* with human NEC, an inflammatory intestinal disorder that affects 2%-5% of neonates and that has very high mortality rates. In mammalian tissue culture, the organism has been shown to adhere to host cells and survive internally in macrophages (Pagotto et al. 2003). However, the specific bacterial virulence factors implicated in the process are still unknown (Mittal et al. 2009). An outer membrane protein A (OmpA) of *C. sakazakii* is also thought to play an important role in the attachment and invasion of Caco-2 cells (Kim et al. 2008). In general, invasion was directly correlated with cell contact time and MOI, suggesting that entry of the bacterium to Caco-2 cells may be receptor mediated and that *C. sakazakii*

invasion is an active process that requires bacterial de novo protein synthesis. The latter might be an important feature in systemic infection of neonates and infants due to their lack of a fully established gut epithelial lining and the normal microbiota (Kim et al. 2008). In contrast, another study demonstrated that *C. sakazakii* binds to rat intestinal epithelial cells without significant invasion (Hunter et al. 2008).

Conclusion

Prebiotic oligosaccharides are now commonly being used as food ingredients in many food products. Several commercial prebiotics have been shown to mediate changes in the intestinal microbiota of the host, in particular, a bifidogenic effect when consumed on a regular basis. Some prebiotics may also be able to directly inhibit infections caused by pathogenic bacteria via an anti-adherence mechanism. This latter activity has led investigators to consider adding anti-adherence prebiotics to powdered infant formula as a way to prophylactically reduce or prevent infections caused by *C. sakazakii*. In addition, the ability of other novel prebiotics and natural plant or milk-derived materials may also have anti-adherent effects against pathogenic bacteria. However, further research will be necessary to determine the feasibility of this approach.

The objectives of the research described in this thesis were to: (1) determine the ability of GOS alone and in combination with other selected commercial prebiotics to inhibit adherence of *C. sakazakii* to tissue culture cells; (2) to determine the ability of plant extracts such as a cranberry extract and

different oligosaccharides to inhibit adherence of enteropathogenic and enterohemorrhagic *Escherichia coli*, and *Salmonella enterica* serovar *Typhimurium* to tissue culture cells; (3) finally, to test the ability of lactoferrin to inhibit adherence of *C. sakazakii*, alone and in combination with two commercially available prebiotics, to tissue culture cells.

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Chapter 2

**Adherence Inhibition of *Cronobacter sakazakii* to intestinal epithelial cells
by prebiotic oligosaccharides.**

**Adherence Inhibition of *Cronobacter sakazakii* to intestinal epithelial cells
by prebiotic oligosaccharides.**

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KEYWORDS: *Cronobacter sakazakii*, *Enterobacter sakazakii*,
galactoligosaccharides, polydextrose, prebiotics, adherence

Abstract

Cronobacter sakazakii is an opportunistic pathogen that has been implicated in meningitis, NEC, and sepsis in neonates. Colonization and subsequent infection and invasion of *C. sakazakii* require that the organism adhere to host cell surfaces. Agents that inhibit or block attachment of the pathogen to epithelial cells could be useful in reducing infections. The goal of this research was to assess the ability of prebiotic galactooligosaccharides (GOS) and polydextrose (PDX) to inhibit adherence of *C. sakazakii* 4603 to a HEp-2 human cell line. Adherence experiments were performed in the presence or absence of prebiotics using HEp-2 cells grown to confluency on glass coverslips. Prebiotics and bacteria were added and incubated for 3 hours. Coverslips were washed and adherence was determined by cultural and microscopic methods. When measured microscopically or by cultural methods, significant reductions in adherence (56% and 71%, respectively) of *C. sakazakii* were observed in the presence of GOS (16 mg/ml). Adherence inhibition also occurred (48%) when a GOS-PDX blend (8 mg/ml each) was tested, although PDX by itself had less effect. Similar results were also observed for Caco-2 cells and also for another strain of *C. sakazakii* (29004). These results suggest that GOS and PDX, alone and in combination, may have an anti-adhesive effect on *C. sakazakii* and directly inhibit the adherence to gastrointestinal epithelial cells.

Introduction

Prebiotics were defined originally as food ingredients that provide beneficial effects for the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon [10]. In recent years, prebiotics have been reported to have additional biological activities, beyond their influence on the gut microbiota. Specifically, it has been suggested that some prebiotics, in particular, galactooligosaccharides (GOS), may be able to inhibit gastrointestinal infections via anti-adhesive activities [9,26,30]. This concept is based on the observation that for most pathogens, the initial adherence to host cell surfaces is one of the first steps prior to colonization and infection. Adherence is most commonly mediated via lectin-like adhesins expressed by the bacteria that recognize and adhere to ligand-like carbohydrates located on the surface of the host epithelial cells. Bacterial variants that are unable to express functional adhesins are unable to adhere and initiate infections, indicating that adherence is required for pathogenesis [1,4,14]. Thus, agents that inhibit adherence could potentially reduce infections [29,35].

The anti-adherence strategy depends on the structural similarity between the carbohydrate binding sites ordinarily recognized by pathogens and the exogenous prebiotic oligosaccharides. The latter would compete with the cognate sugars for binding to the adhesin and thereby inhibit bacterial adherence [27,30]. Indeed, in human milk there are many oligosaccharides that contain residues and linkages similar to those found on the surface of intestinal epithelial cells [18,28]. These human milk oligosaccharides (HMO) have high affinity for

bacterial adhesins, which likely contribute to the anti-adherence properties of human milk. Several recent studies have reported that food-grade oligosaccharides that are currently marketed as prebiotics, also have anti-adhesive activity [27,31,32]. In these studies, food-grade forms of GOS inhibited adherence of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium to the surface of tissue culture cells and also inhibited *Vibrio cholerae* toxin binding to the cell surface toxin receptors. Commercial GOS is comprised of several galactose-containing species of varying lengths, and although this GOS lacks sialic acid and other functional groups ordinarily present in human milk oligosaccharides, at sufficient concentrations they nonetheless have adherence inhibition activity [31]. Another commercial carbohydrate that has been proposed to have prebiotic activity is polydextrose (PDX), a polysaccharide composed of randomly cross-linked glucose units [23]. PDX resists small bowel enzymes and is fermented more slowly by intestinal bacteria versus other non-digestible oligosaccharides [25]. Its anti-adhesive activity, however, has not yet been studied.

Cronobacter sakazakii (formerly *Enterobacter sakazakii*) is now recognized as an emerging opportunistic pathogen and has been implicated in severe forms of meningitis, necrotizing enterocolitis (NEC), and sepsis in neonates [3,16,24]. The infant mortality rate due to infection by this organism has been reported to be 33 – 80%, with up to 20% of surviving neonates developing serious neurological complications [19]. Therefore, prophylactic efforts to mitigate neonatal infections caused by this organism are of considerable interest [11,12].

The main goal of this research, therefore, was to assess the ability of galactooligosaccharide (GOS) and polydextrose (PDX), as well as a blend of GOS and PDX, to inhibit adherence of *C. sakazakii* to a human cell line.

Materials and Methods

Organisms and growth conditions. Strains of *C. sakazakii* used in this study are described in Table 1 and were obtained from K. Venkitanarayanan (Department of Animal Science, University of Connecticut). Prior to each experiment, frozen stock cultures of each organism were thawed, plated onto Tryptic Soy Agar (TSA; Difco) and grown overnight at 37°C. A single colony was inoculated into 10 ml of Tryptic Soy Broth (TSB; Difco) and incubated aerobically, without shaking. Preliminary experiments revealed that adherence rates were highest when cells were in late log phase. Therefore, after 6 hour incubation, cultures were harvested by centrifugation (3,184 x g for 8 minutes). The cells were washed once with phosphate-buffered saline (PBS) and re-suspended in minimal essential medium (MEM; Hyclone, Logan, Utah), supplemented with 10% fetal bovine serum (FBS; Hyclone). Minimal essential medium was pre-equilibrated at tissue culture conditions (5% CO₂, 95% relative humidity, 37°C). Based on additional preliminary experiments, two strains (4603 and 29004) repeatedly gave the highest adherence rates on HEp-2 cells and were used for all subsequent experiments.

Prebiotics (GOS and PDX). Galactooligosaccharide (GOS; Friesland Foods DOMO, Zwolle, the Netherlands) was obtained as a 70% concentrated syrup and

was lyophilized to give a final product of 95% total solids. The GOS concentration, based on the manufacturer's information, was 60% (containing mainly chains of 3-6 monomers in the α -1-4 form), with the balance as lactose, glucose, and galactose. Polydextrose (PDX), obtained from Danisco (New Century, KS), had an average degree of polymerization (DP) of 12 and an average molecular weight of 2000 [7]. The GOS and PDX were prepared as concentrated stock solutions with distilled sterile water at a final concentration of 160 mg/ml. The solutions were filter sterilized using 0.22 μ m filters. A 1:1 blend of PDX and GOS was prepared by mixing equal volumes to give 80 mg/ml of each.

Tissue culture cells. HEp-2 cells were obtained from the American Type Culture Collection (ATCC; Manassas, Virginia). Cells were grown in 75 cm² tissue culture flasks (Corning) containing 25 ml of MEM supplemented with 10% FBS in a CO₂ incubator at tissue culture conditions. Confluent Hep-2 cells were harvested by adding 0.5 ml of 0.25% Trypsin-EDTA Solution (Sigma) and incubating for 15 minutes at tissue culture conditions. Trypsin was inactivated with 0.5 ml of FBS. Cells were then seeded onto 12-mm diameter glass coverslips in 24-well tissue culture plates (Falcon) at approximately 3.6×10^5 viable cells per well, and 500 μ l of MEM supplemented with 10% FBS was added to each well. Plates were incubated under tissue culture conditions for two days prior to the start of each experiment, or until confluency was reached. Media was replaced one day before the experiment. Caco-2 cells (ATCC) were grown as for HEp-2 cells except that the MEM was supplemented with 20% FBS. and the

cells were incubated for fourteen days prior to the start of each experiment, or until confluency was reached. Media was replaced every other day and one day before the experiment.

Adherence Assays. Cell suspensions of *C. sakazakii* 4603 and ATCC 29004 for HEp-2 cells, and *C. sakazakii* ATCC 29004 for Caco-2 cells, were prepared as described above. Prebiotics were mixed with bacterial cultures (in MEM supplemented with FBS) prior to addition to the tissue culture cells. As a control, water was added to the bacterial cultures at the same volume as the prebiotics. The prebiotic solutions represented 10% of the final volume of the suspension, giving a final concentration of 16 mg/ml of prebiotic in the assay mixtures. For the GOS-PDX blend, the final concentration of each prebiotic was 8 mg/ml. The plates were incubated for 3 hours at tissue culture conditions. Preliminary experiments indicated that 3 hours of incubation were optimal for adherence of the bacterial strains to the HEp-2 and Caco-2 tissue culture. The wells were then washed 5 times with PBS to remove non-adhered bacterial cells. HEp-2 experiments were done in duplicate and replicated 5 times (n = 10) for microscopic analyses and 3 times (n = 6) for cultural enumeration. The Caco-2 experiments were done in duplicate and replicated twice (n=4).

Microscopic enumeration. Adhered cells were fixed with 100% methanol for 20 minutes and stained with 10% Geimsa for 15 minutes. The slides were then washed once with sterile distilled water and dried overnight at room temperature. Coverslips were mounted on microscope slides and observed under a phase contrast microscope with the 100x objective. Fifteen pictures of each coverslip

were taken using Motic Image software following an established pattern throughout the entire coverslip. The number of cells and bacteria in each picture were counted using Image J image analysis software. Total adherence was calculated as the number of adhered bacteria per tissue culture cell. Adherence inhibition was calculated as the number of adhered bacteria per cell in the control minus the number of adhered bacteria per cell in the treatment divided by the number of adhered bacteria per cell in the control.

Culture enumeration. Cells were washed as described above and detached by addition of 0.1% Triton X-100 for 30 minutes at room temperature. The cells were collected and enumerated on Tryptic Soy Agar (TSA; Difco) after aerobic incubation at 37°C for 24 hours. Adherence inhibition was calculated as the number of adhered bacteria per ml in the control minus the number of adhered bacteria per ml in the treatment divided by the number of adhered bacteria per ml in the control.

Statistical Analysis. Significant differences between the treatments were determined by analysis of variance (ANOVA) using PROC GLIMMIX in SAS (version 9.2).

Results

GOS and PDX reduce adherence of *C. sakazakii* to HEp-2 cells Adherence assays were conducted for all five strains of *C. sakazakii* on both cell lines (HEp-2 and Caco-2). As other workers previously reported [20], we also observed that some strains had higher adherence rates than others. Specifically, preliminary

experiments (data not shown) showed that strains 4603 and 29004 gave the most consistent results and were used for these studies. Subsequently, adherence of *C. sakazakii* 4603 to HEp-2 cells in the presence and absence of prebiotics was measured by microscopic and culture methods. Both methods revealed that *C. sakazakii* 4603 adherence was significantly reduced when GOS and the GOS-PDX blend was present (Figure 1). By microscopic examination, GOS addition led to a 56% reduction of binding by *C. sakazakii* 4603 to the HEp-2 cells. Similarly, a 48% reduction occurred in the presence of the GOS-PDX blend. However, PDX by itself did not significantly reduce adherence, when measured by microscopy (Figure 1A). Experiments with *C. sakazakii* ATCC 29004 gave similar results when measured microscopically, with reductions of 63% and 53% in the presence of GOS and the GOS-PDX blend, respectively, (Figure 2). However, for this strain, the PDX significantly inhibited (42%) adherence.

The cultural enumeration results confirmed those observed by microscopic examination for both strains (Figures 1B and 2B). However, inhibition levels were somewhat higher when the culture data was used to calculate adherence inhibition. For strain 4603, GOS and the GOS-PDX blend reduced adherence by 71% and 49%, respectively. PDX also inhibited adherence by 55%. The GOS, GOS-PDX, and PDX treatments reduced adherence of strain 29004 by 83%, 80%, and 58%, respectively. Photomicrographs of the coverslips provided additional confirmation that adherence of *C. sakazakii* to HEp-2 cells was reduced in the presence of GOS, PDX and the GOS-PDX blend (Figure 3).

GOS and the GOS-PDX blend also inhibit adherence of *C. sakazakii* to Caco-2 cells. Adherence of *C. sakazakii* ATCC 29004 to Caco-2 cells in the presence and absence of prebiotics was measured by microscopic methods (Figure 4). These experiments revealed that *C. sakazakii* 29004 adherence was significantly lower when the GOS or GOS-PDX blend was present (31 and 38% reductions, respectively). Although a 20% reduction in adherence was observed for the PDX treatment, this reduction was not significant.

Discussion

The use of molecular decoys to reduce pathogen adherence to intestinal epithelial cells and subsequently prevent infection by pathogenic agents was proposed more than a decade ago [35]. However, only recently have food-grade oligosaccharides, including those already used as prebiotics, been shown to have anti-adhesive activity against enteric pathogens [27,31]. In these reports, enteropathogenic *E. coli* and *Salmonella enterica* serovar Typhimurium were inhibited *in vitro* and in mice by two different forms of GOS. These reports led us to consider whether adherence inhibition by prebiotics extended to other enteric pathogens, and *C. sakazakii*, in particular. Although the prevalence of *C. sakazakii* in the environment and in foods is very low and infections are rare, the morbidity rates are very high [8,15]. Therefore, efforts aimed at reducing infections are warranted.

In this study, adherence inhibition of *C. sakazakii* to HEp-2 cells in the presence of prebiotic carbohydrates was determined by two methods, direct microscopic

counting and by cultural enumeration on agar plates. By both methods, the results showed that GOS had an anti-adhesive effect on both of the strains of *C. sakazakii* used in these experiments. The GOS used in these experiments also contained lactose, glucose, and galactose; however, their contribution to adherence was likely not significant, based on previous reports [5,21,31].

Although PDX also had anti-adherence activity, the effects were not significant in all experiments (Figure 1A). In previous experiments, GOS had greater adherence inhibition compared to all other tested oligosaccharides [31].

Similarly, in the experiments with Caco-2 cells, the adherence inhibition of *C. sakazakii* 29004 by GOS was greater than PDX and equal to that of the blend.

Although the pathogenesis of *C. sakazakii* is still under investigation, it appears to share the same initial steps in infection as other pathogens by adhering and colonizing host surfaces [20,22]. It was previously suggested that this organism adheres to host cell tissue by recognizing specific host cell targets [22], indicating that an anti-adherence approach could be useful. However, adherence in *C. sakazakii* was also suggested to be independent of fimbriae [20].

This is the first study in which PDX was compared to GOS in an anti-adherence model. Previously, an *in vitro* analysis suggested that GOS is fermented primarily in the proximal end of the large intestine, whereas PDX fermentation occurs from the proximal to the distal end [13,25]. The fermentation of PDX resulted in lower levels of short chain fatty acids (SCFA) compared to GOS, suggesting that PDX may be less bifidogenic. In another *in vitro* study, PDX resulted in lower *E. coli* counts, compared to other prebiotics, although the

mechanism was not determined [33]. In adults, consumption of PDX improved bowel functions, produced SCFAs, and promoted the proliferation of a favorable intestinal microbiota [17]. Similarly, in an infant feeding trial with GOS and PDX, the stool characteristics of infants receiving the prebiotic mixture were more similar to those of breast-fed infants, in comparison to infants fed unsupplemented formula [34].

Our results showed that PDX inhibited adherence of *C. sakazakii* on HEp-2 cells, although it was generally less inhibitory than GOS, at the same concentration. On Caco-2 cells, the PDX-GOS blend, each at 8 mg/mL, was as inhibitory as the GOS alone (at 16 mg/mL), suggesting that additional research on the anti-adherence properties of PDX might be warranted. Mange et al. [20] examined the adherence properties of 50 *C. sakazakii* strains on three cell lines, including HEp-2 and Caco-2 and reported that considerable heterogeneity existed within strains. While mannose reduced diffuse adherence, cluster-mediated adherence was unaffected. Thus, the specific means by which adherence occurred could not be established. Apart from the differences between cells lines, the adherence inhibition results seen in our study are also likely due to structural differences between the galactose-containing GOS and the glucose-containing PDX.

Conclusions

Collectively, our results showed that GOS and GOS-PDX blends effectively reduced adherence of *C. sakazakii* in tissue culture experiments. Although GOS

is chemically distinct from the human milk oligosaccharides that might be expected to have even greater adherence inhibition [2,18,28], there is evidently enough similarity between GOS and human milk oligosaccharides to be effective at the concentrations used in this study. Consequently, *in vivo* supplementation with GOS or GOS-PDX blends might parallel some of the functionalities of these milk oligosaccharides. However, it should be noted that the concentration of prebiotics used in this *in vitro* study exceeds the supplementation level generally reported for commercial infant formula [6].

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Figure 1. Adherence of *C. sakazakii* 4603 to Hep-2 cells in the presence of prebiotics (16 mg/ml). In A, adherence was measured by microscopic counting (n = 10), and in B, adherence was measured by cultural enumeration (n = 6). Statistically significant effects ($p < 0.05$) are indicated by the asterisk.

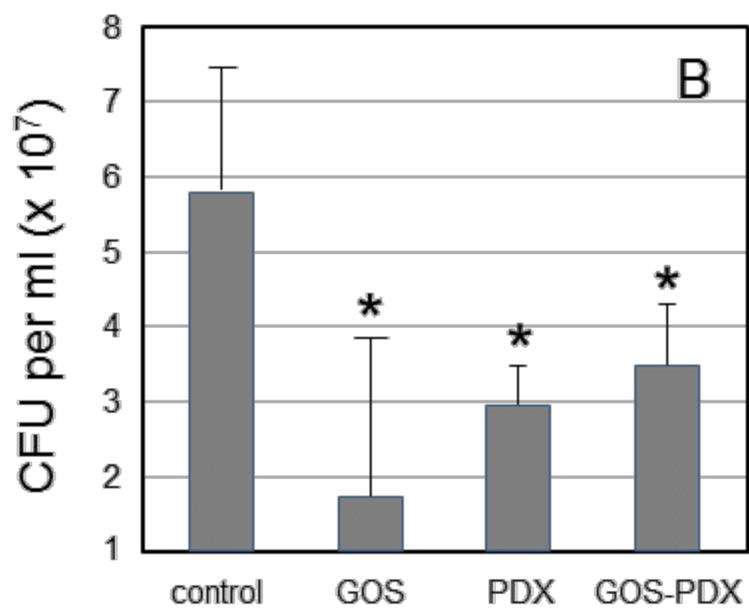
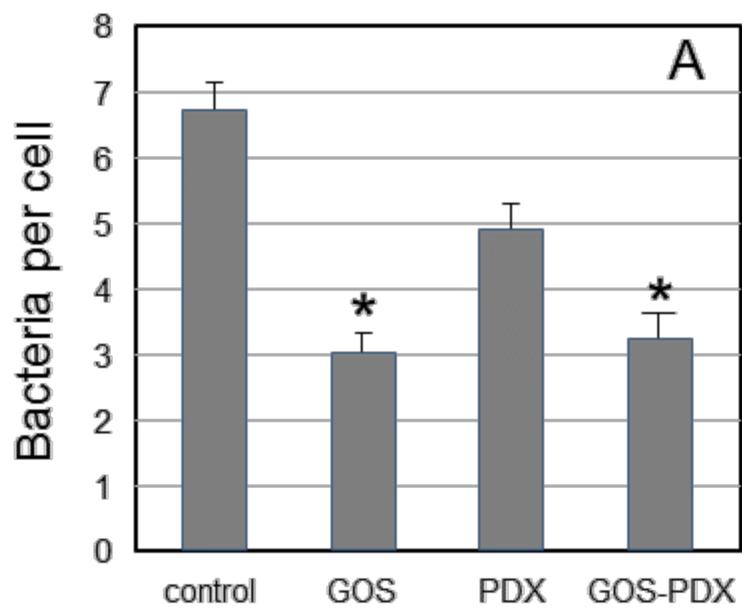


Figure 2. Adherence of *C. sakazakii* 29004 to Hep-2 cells in the presence of prebiotics (16 mg/ml). In A, adherence was measured by microscopic counting (n = 10), and in B, adherence was measured by cultural enumeration (n = 6). Statistically significant effects ($p < 0.05$) are indicated by the asterisk.

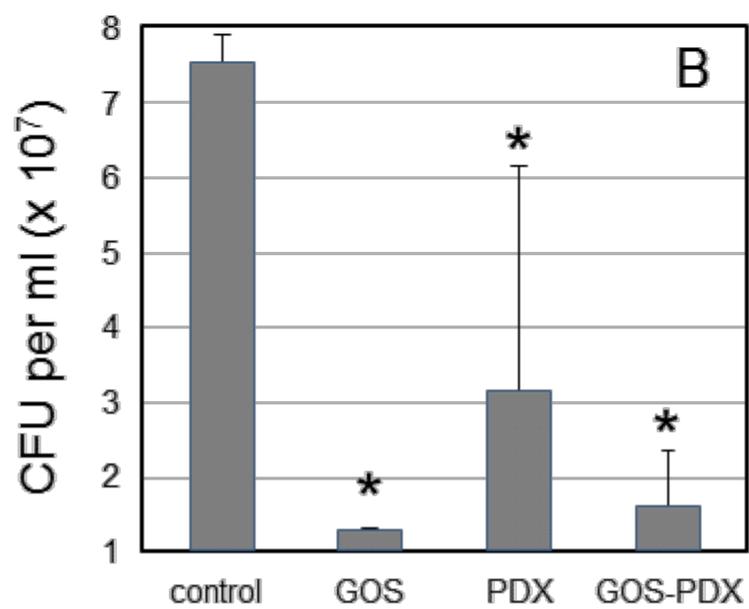
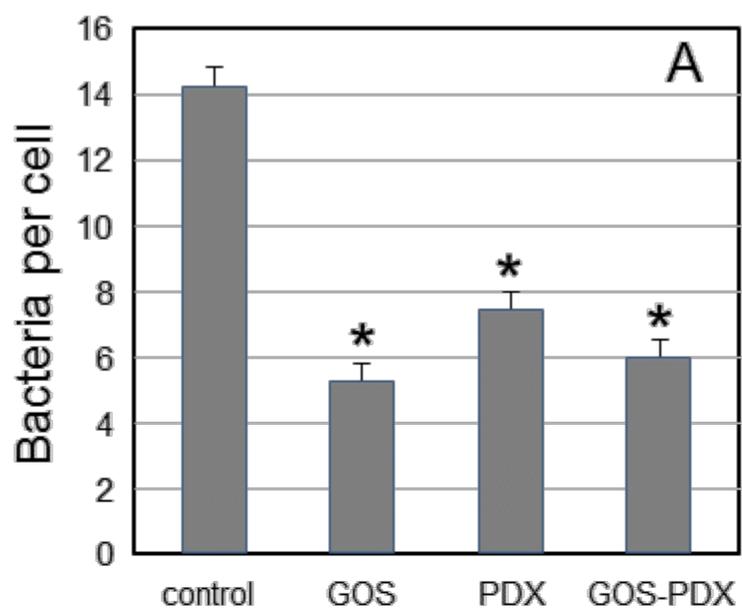


Figure 3. Adherence of *C. sakazakii* 4603 to Hep-2 cells in the absence (A) and presence of GOS (B), GOS-PDX (C); and PDX (D).

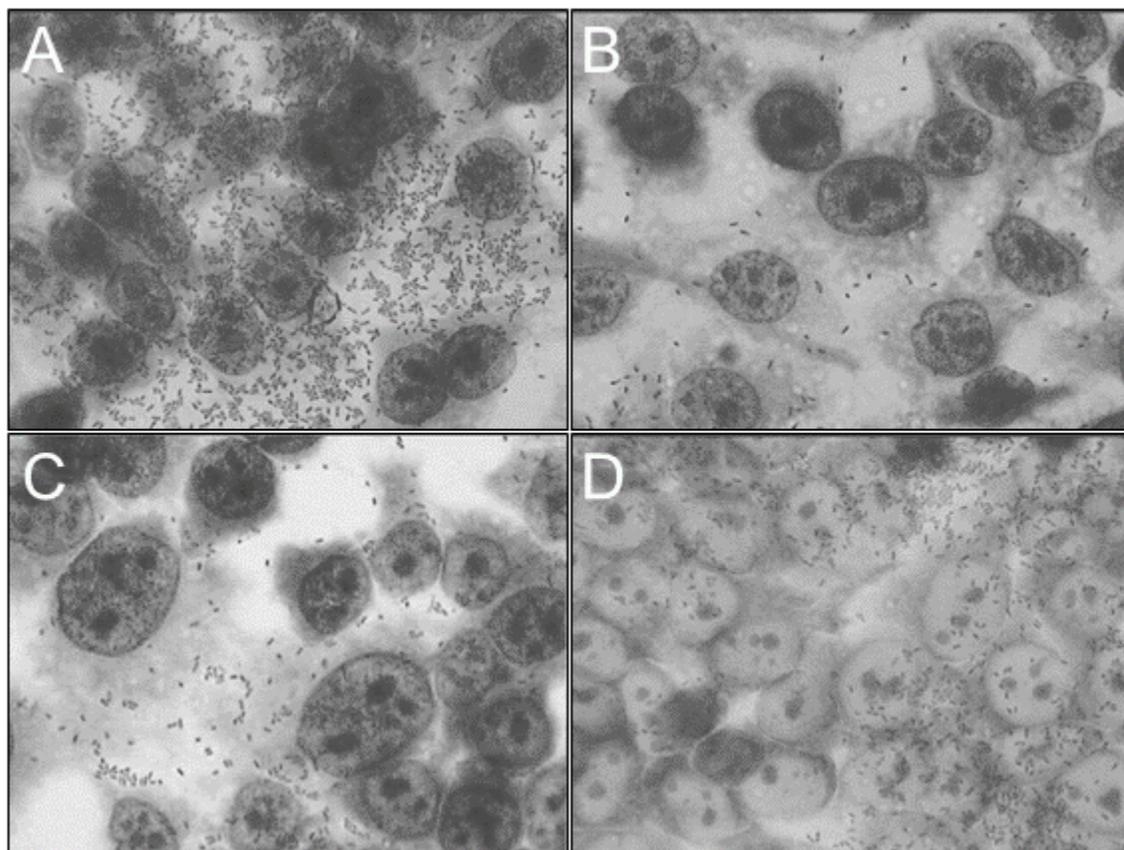


Figure 4. Adherence of *C. sakazakii* 29004 to Caco-2 cells in the presence of prebiotics (16 mg/ml). Adherence was measured by microscopic counting (n = 4) at 1000 x magnification. Significant effects (versus the control) are indicated by the asterisk ($p < 0.05$).

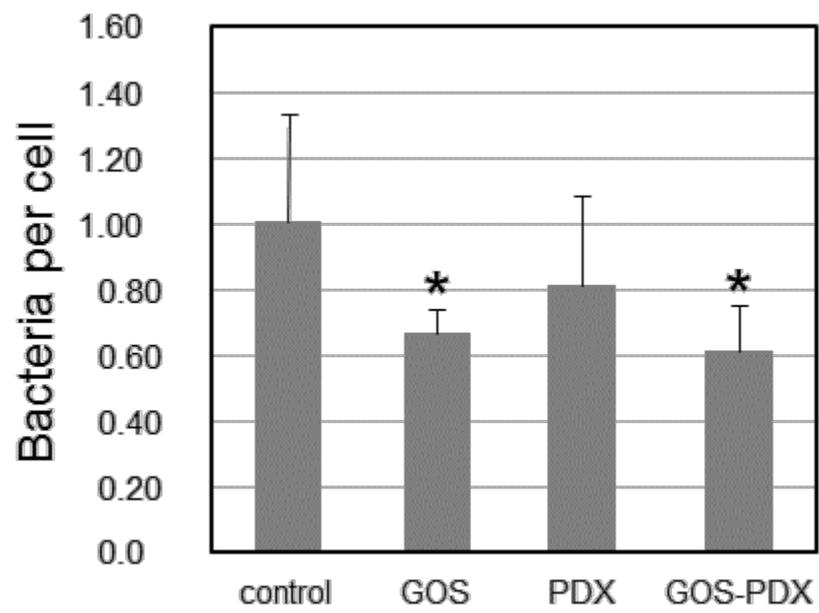


Table 1. Strains and Sources

Strain	Source
<i>Cronobacter sakazakii</i> 4593	Milk powder isolate (infant formula)
<i>Cronobacter sakazakii</i> 4603	Milk powder isolate (infant formula)
<i>Cronobacter sakazakii</i> 29004	ATCC (deposited by CDC)
<i>Cronobacter sakazakii</i> 4583	Enterocolitis isolate
<i>Cronobacter sakazakii</i> 415	Meningitis isolate

Chapter 3

Adherence Inhibition of Intestinal Pathogens by Prebiotic Oligosaccharides and Plant Extracts

Abstract

Although prebiotic oligosaccharides are well known for their ability to modulate the intestinal microbiota, they have been shown to have other biological activities. Specifically, oligosaccharides and plant extracts may also act as molecular decoys that inhibit adherence of pathogens to the surface of epithelial cells. The goal of this research was to assess the ability of several prebiotic agents, including chitooligosaccharides (COS) and mannan oligosaccharides derived from yeast cell walls (MOSy) and konjac root (MOSk), to inhibit adherence of enteropathogenic *Escherichia coli* (EPEC), enterohemorrhagic *Escherichia coli* (EHEC) and *Salmonella typhimurium* to a human HEp-2 cell line. In addition, a high molecular weight component of cranberry (CHMW) was also assessed for anti-adherence. Different fractions of COS significantly reduced adherence of EPEC at a concentration of 16 mg/ml. Although MOSy inhibited EPEC, EHEC, and *S. typhimurium*, adherence inhibition was not observed for MOSk. Adherence inhibition of EPEC, EHEC, and *S. typhimurium* by CHMW was observed. These results suggest that different oligosaccharides and plant extracts may be promising therapeutic agents that could be added to foods in order to prevent or mitigate bacterial adhesion to gastrointestinal epithelial cells.

Introduction

Prebiotics were originally defined more than 15 years ago as “non-digestible food ingredient(s) that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the

colon, and thus improves host health” (Gibson et al. 1995). Although this definition has been modified slightly (M. Roberfroid 2007), to consider a substance as a prebiotic still depends on the ability of that substance to effect positive changes in the gut microbiota. Recently, however, some prebiotics have been shown to possess another quite different biological activity that also contributes to the health of the host. Specifically, certain prebiotic oligosaccharides are able to interfere with the adherence mechanism used by pathogenic bacteria to attach to the surface of target tissues, in the gut as well as in extraintestinal tissues (Shoaf et al. 2006; Kunz et al. 2000). The model is based on the structural similarity that different oligosaccharides have to the receptors recognized by pathogens as binding sites. Since adherence is the first and perhaps rate-limiting step in many bacterial infections, strategies based on preventing or inhibiting adherence could be effective at reducing infections and the subsequent onset of disease (Klemm et al. 2010; Bavington et al. 2005; Shoaf et al. 2006).

Several commercial prebiotics have been assessed in vitro for their ability to inhibit pathogen adherence to epithelial cells, including inulin, galactooligosaccharides (GOS), and fructooligosaccharides (FOS). In particular, GOS has been reported to inhibit adherence of *Escherichia coli*, *Salmonella enterica* serovar *Typhimurium* and two different strains of *Cronobacter sakazakii* to tissue culture cells (Shoaf et al. 2006; Quintero et al. 2011; McGowan et al. 2011, manuscript in preparation). In addition, other putative prebiotics extracted from plant material have also been reported to have anti-adherence activity,

including pectic oligosaccharides that were shown to reduce invasion of *Campylobacter jejuni* to a Caco-2 cell line (Ganan et al. 2009).

One group of plant extracts that has long been studied for their ability to prevent infections are cranberry extracts. Juices and cocktails obtained from cranberries have been consumed for decades with the purpose of treating and preventing urinary tract infections (Schaeffer et al. 2004). The high molecular weight component of cranberry juice has been reported to inhibit the adhesion of a mannose specific Type 1 fimbriated *E. coli* to tissue culture cells (Zafriri et al. 1989). Additionally, several studies have reported that cranberry juice constituents inhibit adherence of *Helicobacter pylori* to the surface of epithelial cells *in vitro* and *in vivo*. (Burger et al. 2000; Burger et al. 2002; Shmueli et al. 2004). Furthermore, cranberry has been shown to inhibit the adhesion of oral bacteria such as *Streptococcus mutans* onto tooth surfaces (Steinberg et al. 2005; 2004; Weiss et al. 2004).

Infections caused by enteric pathogens are commonly treated with antibiotics. Although antibiotics have been very effective at reducing mortality caused by bacterial infections, their use has also led to the appearance of resistant strains (Davies et al. 2010). Therefore, efforts to develop new approaches for preventing infections are a high priority among public health experts. In this research, several different oligosaccharides and plant extracts were evaluated for their ability to inhibit adherence of selected bacterial pathogens to HEP-2 tissue culture cells.

Materials and Methods

Organisms and growth conditions

Enteropathogenic *Escherichia coli* (EPEC) EPEC strain E2348/69 (O127:H6) was obtained from M. Donnenberg (University of Maryland School of Medicine, Baltimore). Before each experiment, frozen stock cultures of were plated onto Tryptic Soy Agar (TSA; Difco) and grown overnight at 37°C. A single colony was inoculated into 10 ml of Tryptic Soy Broth (TSB; Difco) and incubated overnight at 37°C without shaking. Overnight cultures were then inoculated at 1% (vol/vol) into Modified Eagle Medium (MEM; Hyclone) supplemented with 10% fetal bovine serum (FBS; Hyclone) that was pre-equilibrated at tissue culture conditions (5% CO₂, 95% relative humidity, 37°C). Cells were then incubated in MEM for 80 min at 37°C prior to the start of the experiment.

Salmonella enterica* serovar *Typhimurium Before each experiment, frozen stock cultures of were plated onto Luria Bretani Agar (LB; Difco) and grown overnight at 37°C. A single colony was inoculated into 10 ml of Luria Bretani Broth (LB; Difco) and incubated for 23-24 hours at 37°C without shaking. Cultures were then washed once with phosphate buffer saline (PBS) and resuspended in pre-equilibrated MEM supplemented with 10% FBS.

Enterohemorrhagic *Escherichia coli* (EHEC) EHEC strain ATCC 43888 (O157:H7) was obtained from the American Type Culture Collection (ATCC; Manassas, Virginia) and the same procedure for EPEC was followed.

Prebiotics and Plant Extracts

Cranberry. Cranberry concentrate was obtained from Ocean Spray Cranberries, Inc. The concentrate was used directly as concentrated material following neutralization with 1M NaOH. The total solids concentration was determined on a dry weight basis. The cranberry concentrate was also fractionated by dialysis to yield a high molecular weight component (HMW). Exhaustively dialysis was performed for 5 days at 4°C using 12,000 – 14,000 MW cut-off dialysis tubing against distilled water. The non-dialyzable material was then freeze dried. A stock solution was prepared by resuspending the freeze dried material in distilled water to a concentration of 10 mg/ml. The solution was filter sterilized using 0.22-µm filters.

Chitin Oligosaccharides (COS). Different COS fractions (based on MW) were obtained from Berit Bjugan Aam (Norwegian University of Life Science, Norway), as freeze dried powders. Freeze dried material was resuspended in autoclaved distilled water and final concentration of the stock solution was determined for each fraction.

Konjac Mannan (MOSk). Konjac mannan was obtained from Wayne Muller (United States Army NSRDEC, Natick, MA) as a dry powder. MOSk was prepared as a concentrated stock solution with distilled water at a final concentration of 160 mg/ml. The solution was filter sterilized using 0.22-µm filters.

Yeast Mannan (MOSy) Yeast mannan was obtained from Lallemand (Ontario, Canada) as a powdered material. A stock solution was prepared dissolving 160 mg of powder per ml of distilled water. The solution was centrifuged at 201 x g for 10 minutes and the supernatant was saved. The supernatant was filtered through a 0.4- μ m filter and subsequently freeze dried. A stock solution was prepared in distilled water at a final concentration of 200 mg/ml.

Tissue culture cells HEp-2 cells were obtained from ATCC. Cells were grown in 75 cm² tissue culture flasks (Corning) containing 25 ml of MEM supplemented with 10% FBS in a CO₂ incubator at tissue culture conditions. Confluent Hep-2 cells were harvested by adding 0.5 ml of 0.25% Trypsin-EDTA Solution (Sigma) and incubating for 15 minutes at tissue culture conditions. Trypsin was inactivated with 0.5 ml of FBS. Cells were then seeded onto 12-mm diameter glass coverslips in 24-well tissue culture plates (Falcon) at approximately 3.6 x 10⁵ viable cells per well, and 500 μ l of MEM supplemented with 10% FBS was added to each well. Plates were incubated under tissue culture conditions for 30 hours prior to the start of each experiment, or until confluency was reached.

Adherence Assays Cell suspensions of EPEC E2348/69 (O127:H6), *Salmonella enterica* serovar *Typhimurium*, and EHEC were prepared as described above. Prebiotics were mixed with bacterial cultures (in MEM supplemented with FBS) prior to addition to the tissue culture cells. As a control, water was added to the bacterial cultures at the same volume as the prebiotics. The prebiotic solutions were prepared at different concentrations depending on the product used. The plates were incubated for 30 minutes for EPEC E2348/69 (O127:H6) and

Salmonella enterica serovar *Typhimurium*, and 90 minutes for EHEC. The wells were then washed 5 times with PBS to remove non-adhered bacterial cells. Experiments were done in duplicate and replicated 3 times (n=6) for analysis with quantitative real time PCR (qRT-PCR) and 2 times for microscopic analyses (n=4).

Quantitative Real Time PCR (qRT-PCR). Bacterial cells from adherence assays were detached by the addition of 1 ml of 0.1% Triton X-100 for 30 minutes at room temperature. Bacterial cells were harvested and centrifuged for 5 min at 10,000 x g, supernatant was discarded and pellet was resuspended in 180 µl of buffer ATL from the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Instructions provided in the manufacturer's manual for DNA extraction of Gram negative bacteria were followed to complete the extraction.

Quantitative real time PCR (qRT-PCR) was performed using a Mastercycler Realplex2 (Eppendorf AG, Hamburg, Germany). Each PCR was done in a 25 µl volume. The reaction mixture comprised 11.25 µl of the 20x SYBR solution and 2.5-µl Real-MasterMix (5Prime), 0.5 µM of each primer, and 1 µl of DNA template. (Martínez et al. 2009) Specific primers and amplification program were followed for each organism.

Table 1. Primers and PCR programs for the different organisms used in this study.

Organism	Primers (5' - 3')	Amplification Program
EPEC and EHEC	eaeFor GGCGATTACGCGAAAGATAC eaeRev GATTAACCTATGCCGTTCCA	Initial denaturation at 95°C for 10 min and 40 cycles at 95°C for 15s and 62°C for 1 min for annealing and extension.
Salmonella	139 -GTGAAATTATCGCCACGTTCCGGGCAA 141 -TCATCGCACCGTCAAAGGAACC	Initial denaturation at 94°C for 2 min and 45 cycles at 94°C for 20s and 62°C for 1 min for annealing and extension

Microscopic enumeration Adhered cells were fixed with 100% methanol for 20 minutes and stained with 10% Geimsa for 15 minutes. The slides were then washed once with sterile distilled water and dried overnight at room temperature. Coverslips were mounted on microscope slides and observed under a phase contrast microscope with the 100x objective. Fifteen pictures of each coverslip were taken using Motic Image software following a pre-established pattern throughout the entire coverslip. The number of cells and bacteria in each picture were counted using Image J image analysis software. Total adherence was calculated as the number of adhered bacteria per tissue culture cell. Adherence inhibition was calculated as the number of adhered bacteria per cell in the control minus the number of adhered bacteria per cell in the treatment divided by the number of adhered bacteria per cell in the control.

Statistical analysis Significant differences between the treatments were determined by one way analysis of variance (ANOVA) using Tukey to compare all pairs of columns with $\alpha = 0.05$. GraphPad Prism5 software was used to perform statistical analysis.

Results

Cranberry concentrate reduces adherence of EPEC and *Salmonella*

***Typhimurium* to HEp-2 cells.** Adherence of EPEC and *Salmonella typhimurium* in the presence and absence of a neutralized cranberry concentrate to HEp-2 cells was measured by qRT-PCR (Figure 2). Experiments revealed that the neutralized cranberry concentrate inhibited adherence of EPEC and *S. typhimurium* at a minimum concentration of 25 and 10 mg/ml, respectively.

Cranberry High Molecular Weight Component reduces adherence of EPEC, EHEC, and *Salmonella Typhimurium* to HEp-2 cells. Adherence of EPEC, EHEC and *Salmonella Typhimurium* in the presence and absence of a cranberry HMW component to HEp-2 cells was measured by qRT-PCR and by microscopic enumeration (Figure 1). Both methods revealed that the HMW component of cranberry inhibits adherence of EPEC (A,B), *Salmonella typhimurium* (C, D), and EHEC. (E, F)

COS inhibits adherence of EPEC to HEp-2 cells. Adherence of EPEC in the presence and absence of different fractions of chitin oligosaccharides to HEp-2 cells was measured by microscopic examination (Figure 3). At a concentration of 16 mg/ml, COS fraction KN + C88 (A) inhibited adherence by 93%, fraction

W275A (C) reduced adherence by 72%, FA 0.15(NC) reduced adherence by 76% and fraction FA 0.61 reduced adherence by 74%.

MOSk does not inhibit adherence of EPEC, EHEC, and *Salmonella*

***Typhimurium* to HEp-2 cells.** Adherence of EPEC, EHEC, and *Salmonella typhimurium* in the presence and absence of MOSk to HEp-2 cells was measured by qRT-PCR and microscopic counting (Figure 4). Both methods revealed there was no inhibition of EPEC, EHEC, or *Salmonella typhimurium* by MOSk.

MOSy inhibits adherence of EPEC and *Salmonella Typhimurium* to HEp-2

cells. Adherence of EPEC, EHEC and *Salmonella typhimurium* in the presence and absence of MOSy to HEp-2 cells was measured by qRT-PCR and microscopic examination (Figure 5). Both methods revealed that MOSy inhibits adherence of EPEC and *Salmonella typhimurium*. Results show no significant reduction in adherence of EHEC to HEp-2 cells.

Discussion

The use of molecular decoys to prevent pathogen adherence was proposed more than a decade ago (Ebrahim 1997; Andersson et al. 1986; Cravioto et al. 1991; Ofek et al. 1978). In recent years, several oligosaccharides, including food and non-food grade materials, have been tested for their anti-adherence effects against different pathogens. Extracts from plants and other natural sources have also been studied for their anti-adherence activities. In this report, we compared five different novel oligosaccharides and plant extracts

for their ability to prevent or inhibit adherence of EPEC, EHEC, and *S. typhimurium* to the surface of epithelial cells. The results showed that while some of the test materials were very effective, other agents had little ability to inhibit adherence.

Cranberry juice consumption has long been promoted for its putative therapeutic activities, and especially for preventing or treating urinary tract infections (Schaeffer 2004). It was reported that the maximum adherence inhibition of *Helicobacter pylori* to gastric mucus was at a concentration of 1 mg/ml (Burger et al. 2000). Similarly, it was also reported that cranberry juice cocktail reduced adherence of *E. coli* isolates from patients with UTI by more than 75% (Sobota 1984). The mechanism by which cranberry inhibits adhesion is poorly understood, although it has been reported that the high molecular weight component might directly interact with P-fimbriae by altering the latter's conformation and also by binding hydrophilic components (Y. Liu et al. 2006). Our results when using neutralized cranberry concentrate and the HMW component show inhibition of enteric pathogens such as *Salmonella typhimurium*, EPEC and EHEC to intestinal epithelial cells. These results suggest that consumption of cranberry juice might be helpful in preventing infections caused by the latter organisms.

Chitosan derivatives have several applications in the food industry, and one of the most important properties is their antimicrobial activity; (Xia et al. 2011; Tsai et al. 2002) the latter has been reported to be, to a certain extent, by inhibiting bacterial adherence. (Rhoades et al. 2006; Xia et al. 2011) Four

different fractions of chitin oligosaccharides were tested in this study; they all showed bacterial adherence inhibition higher than 70%. Growth of the organism in TSB with COS at the same concentration was also measured to determine if COS had a general bactericidal effect; no inhibition was observed (data not shown). Thus, COS appears to have significant antiadherent activity, although further research is needed to establish how stable and safe this material is before it could be added as a food ingredient.

Mannan from konjac root did not inhibit adherence of any of the organisms studied. Bacterial adherence can be mediated by specific proteins present in the surface of bacteria, which recognize specific receptors on the surface of epithelial cells. (Bavington et al. 2005; Klemm et al. 2010; Sharon 2006) The specificity of these lectin-like adhesins depends greatly on the bacterial species and may vary greatly among organisms, even among bacterial strains. For this reason, not all the proposed oligosaccharides or plant extracts will necessarily inhibit pathogen adherence to the surface of epithelial cells.

Mannan oligosaccharides (MOS) from yeast cell walls have been shown to be important in the reduction of pathogen colonization (K. Newman et al. 1994). Many gram-negative bacteria attach to the surface of epithelial cells via mannose-specific fimbriae (Ofek et al. 1977); MOS can act as a molecular decoy to inhibit adherence of these pathogens to epithelial cells. Spring et al. 2000 reported that multiple strains of *Salmonella* and *Escherichia coli* agglutinated MOS *in vitro*. In this study, mannan obtained from the cell wall of yeast showed inhibition of different bacterial strains to epithelial cells.

Conclusions

Collectively, our results showed that different oligosaccharides and plant extracts reduce adherence of *Escherichia coli* and *Salmonella typhimurium* in tissue culture cells. However, not all of the oligosaccharides tested were effective in reducing adherence, suggesting that binding inhibition depends on the adherence mechanism of the organism and the specific structure of the oligosaccharide. Further research is needed to determine whether the compounds tested can be food grade and if the concentration at which they inhibit is within the economic and functional ranges suitable for food products.

Acknowledgements

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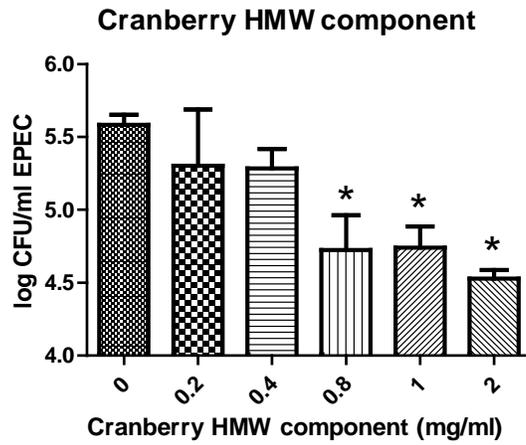
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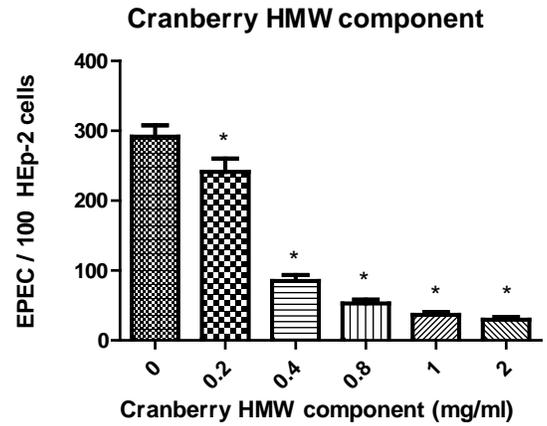
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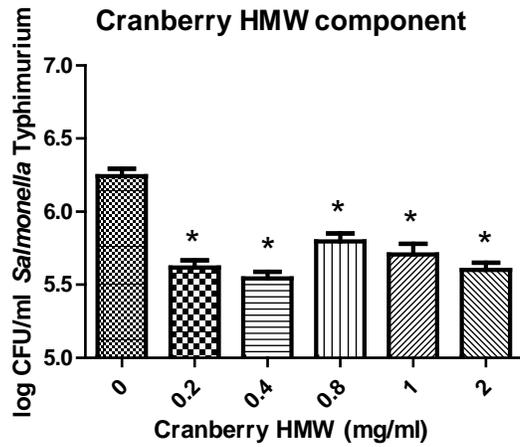
Figure 1. Adherence of EPEC, EHEC, and *Salmonella typhimurium* to HEp-2 cells in the presence of a cranberry HMW component (0, 0.2, 0.4, 0.8, 1, and 2 mg/ml) analyzed by qRT-PCR (n=6) (A, C) and microscopic counting (n=4) (B, D, F). Statistically significant effects are indicated by an asterisk.



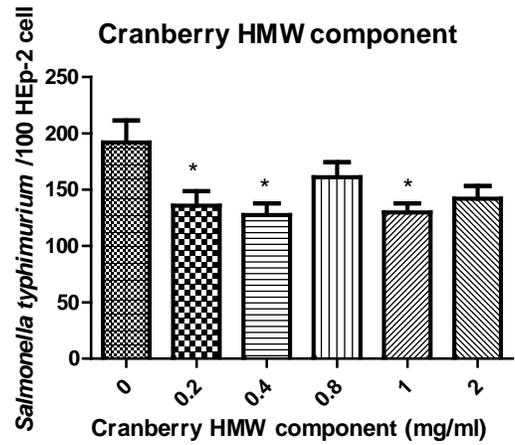
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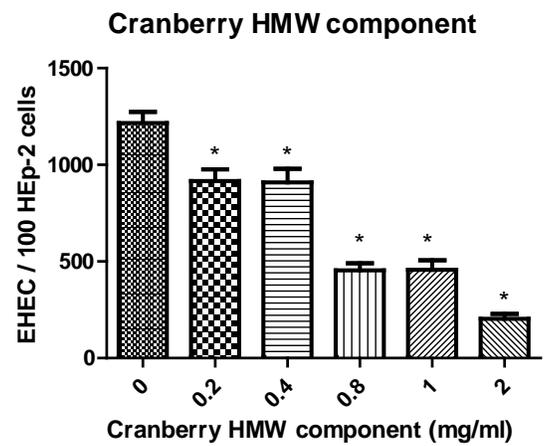
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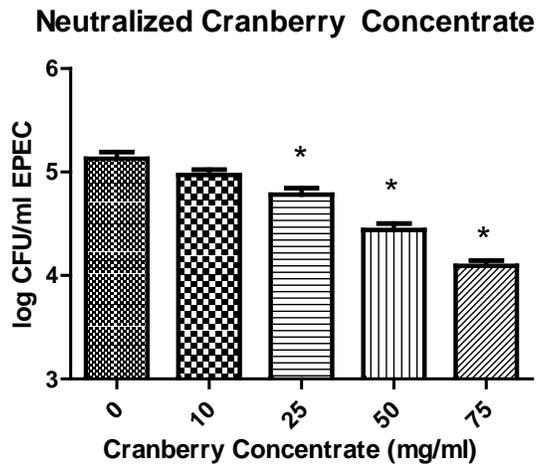
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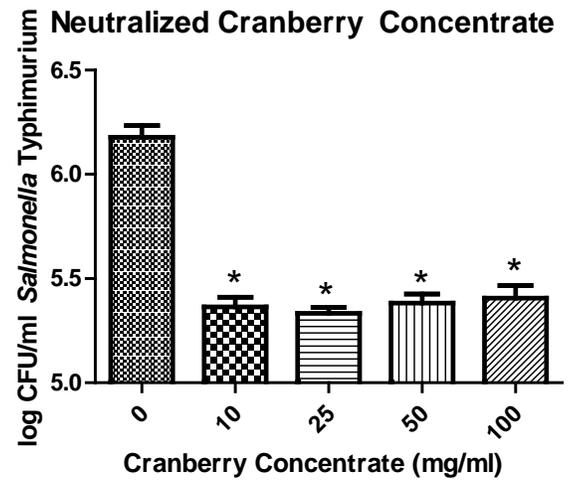
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Figure 2. Adherence of EPEC and *Salmonella typhimurium* to HEp-2 cells in the presence of a neutralized cranberry concentrate analyzed by qRT-PCR (n=6).

Statistically significant effects are indicated by an asterisk.



A



B

Figure 3. Adherence of EPEC to HEp-2 cells in the presence of COS analyzed by microscopic enumeration. (n=6) Statistically significant effects are indicated by an asterisk.

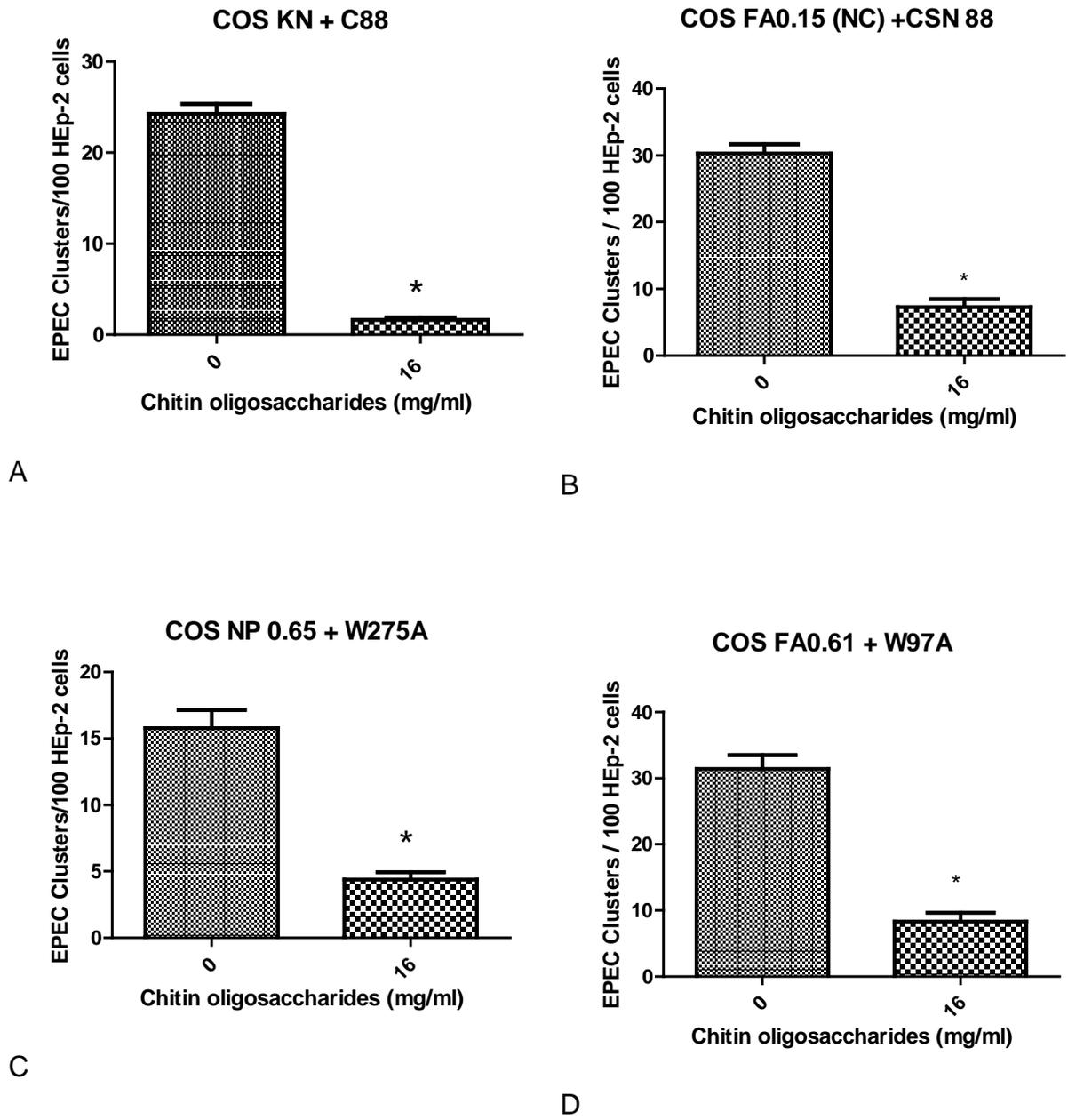
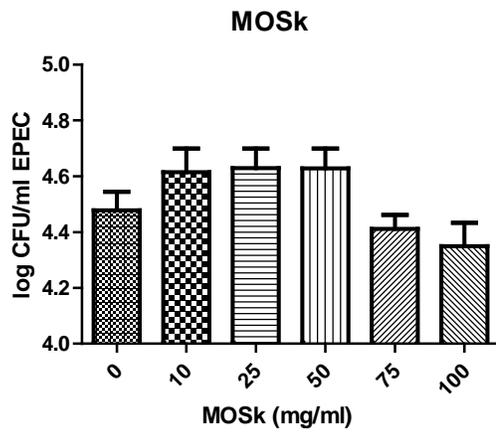
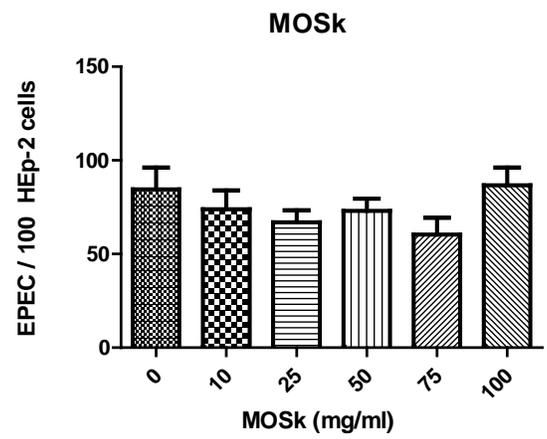


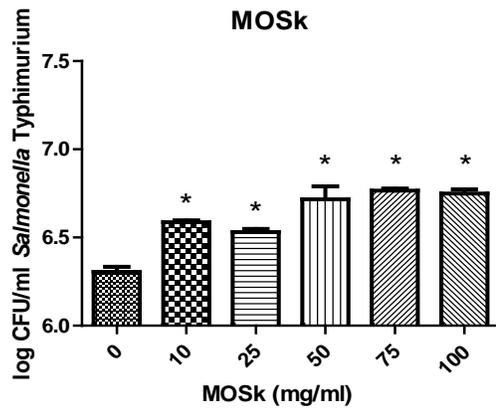
Figure 4. Adherence of EPEC, EHEC, and *Salmonella typhimurium* to HEp-2 cells in the presence of MOSk analyzed by qRT-PCR (n=6) (A, C, D) and microscopic counting (n=4) (B). Statistically significant effects are indicated by an asterisk.



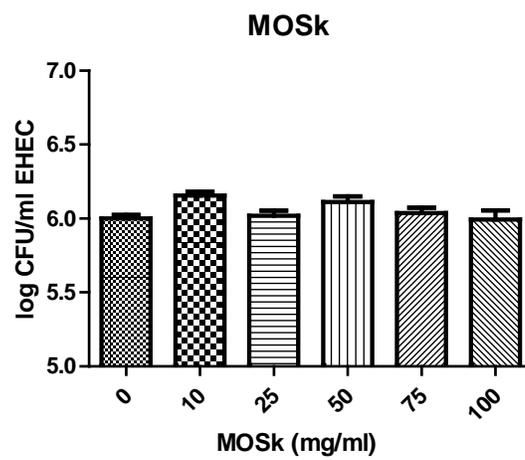
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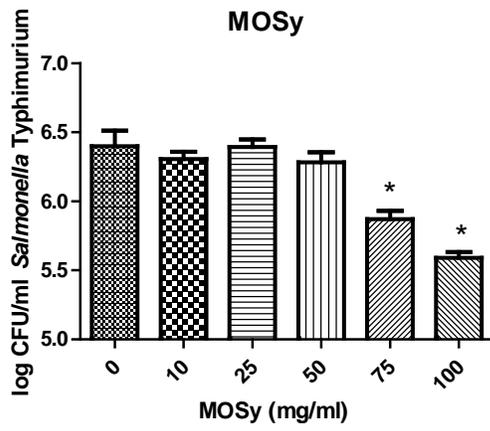


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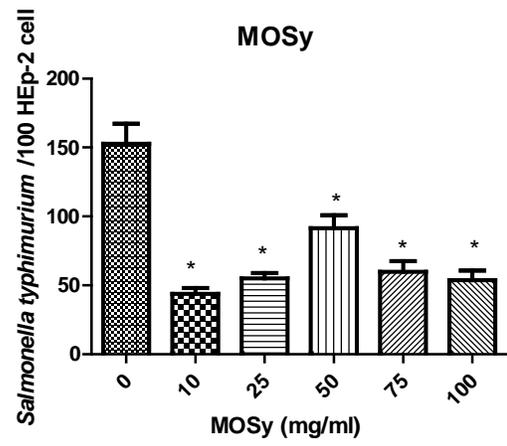


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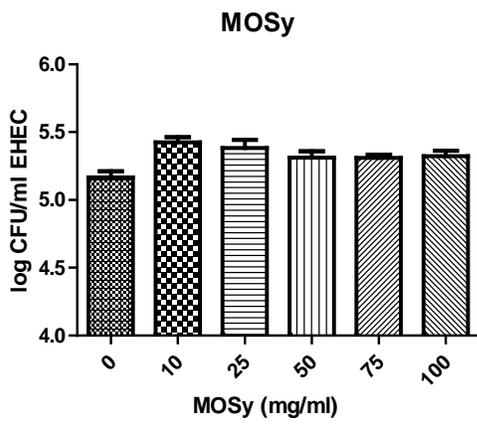
Figure 5. Adherence of EPEC, EHEC, and *Salmonella typhimurium* to HEp-2 cells in the presence of MOSy by qRT-PCR (A, C, D) (n=6) and microscopic counting (B) (n=4). Statistically significant effects are indicated by an asterisk.



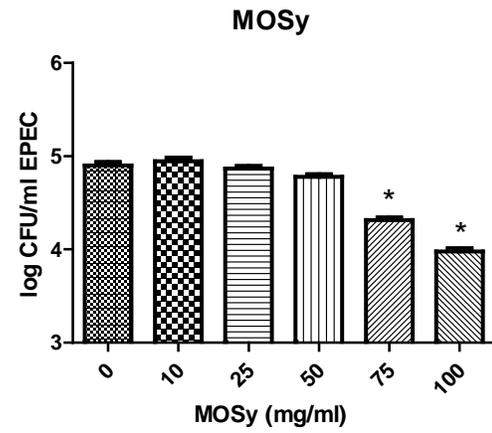
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Chapter 4

**Lactoferrin as an anti-adherent agent: Inhibition of *Cronobacter sakazakii*
adherence to epithelial cells**

Abstract

Cronobacter sakazakii is now recognized as an opportunistic pathogen and has been implicated in rare but severe cases of NEC, meningitis, and sepsis in neonates. The first step in bacterial pathogenesis requires the organism to adhere to host cells surfaces; therefore, agents that inhibit adhesion might be useful for preventing infections. Lactoferrin, an iron binding protein found in milk, has been shown to inhibit bacterial adherence by direct interaction and disruption of bacterial surfaces. Therefore, the goal of this research was to assess the ability of two different types of lactoferrin to inhibit adherence of *Cronobacter sakazakii* to a HEp-2 human cell line. Results showed that the adherence of *C. sakazakii* was significantly reduced at a minimum concentration of 10 mg/ml. However, at higher concentrations (up to 50 mg/ml), further reductions in adherence were not observed. These results suggest that lactoferrin might interact with *C. sakazakii* and directly inhibit adhesion to tissue culture cells.

Introduction

Lactoferrin (Lf) is an 80 kDa iron binding glycoprotein, belonging to the transferrin family. It is found in high concentrations, up to to 7 g/L in human colostrum (Actor et al. 2009) and up to 1.5 mg/ml in bovine colostrum (Yekta et al. 2010). It is also present in fluids of the digestive tract (Giugliano et al. 1995; Legrand et al. 2005). The concentration of lactoferrin in milk varies over time and is dependent on the stage of lactation (Farnaud 2003).

Several biological functions have been attributed to lactoferrin. It has bactericidal and bacteriostatic properties, as well as anti-inflammatory and immunomodulatory activities, based on both in-vitro and in-vivo models (Gomez et al. 2003). In particular, lactoferrin is known for its antimicrobial activity, for which at least two different mechanisms have been proposed. The first model is based on the ability of LF to bind iron, hence depriving pathogens from essential nutrients and thereby inhibiting their growth (Farnaud 2003; Jenssen et al. 2009). This antimicrobial activity is reduced upon iron saturation of the molecule, suggesting that LF is bacteriostatic rather than bactericidal (Farnaud 2003; Arnold et al. 1980; Kalmar et al. 1988; Yamauchi et al. 1993; Valenti et al. 2005). A second means by which LF exhibits antimicrobial activity is via an iron-independent mechanism. According to this model, LF inhibits bacterial pathogens by a direct interaction mediated by binding of the Lipid A portion of the lipopolysaccharide (LPS) of Gram negative bacteria (Appelmek et al. 1994; Brandenburg et al. 2001; Shahriar et al. 2006). This then leads to the disruption of the bacterial membrane and results in the release of LPS (Ellison et al. 1988; Appelmek et al. 1994; Brandenburg et al. 2001).

There is now evidence that lactoferrin can also inhibit bacterial adherence to host cell surfaces, which is the first step for bacterial pathogenesis. It is well known that many enteric pathogens, including enteropathogenic *E. coli* (EPEC), adhere to and infect host cells via a Type III secretion system. By blocking the initial step of EPEC attachment to host cells, the actin polymerization step of the TTSS cascade is impaired (Ochoa et al. 2003). Other investigations (Qiu 1998)

demonstrated that human lactoferrin also has proteolytic activity, hence providing an additional mechanism by which it can exhibit anti-adherent activity (Valenti et al. 2005). In addition, it was also reported (Yekta et al. 2010) that lactoferrin inhibits adherence of *E. coli* O157:H7 to Caco-2 cells in a dose dependant manner, mainly due to disruption of the Type III Secretory System.

In addition to its anti-adherence role, Lactoferrin has been identified as a critical component for mediating immune responses. It reduces oxidative stress, thus controlling excess inflammatory response (Actor et al. 2009). It may limit the inflammation caused by bacterial infections, which can lead to prevention of septic shock (Legrand et al. 2008). Along with its anti-inflammatory properties, it is involved in many events related to immune responses, such as modulation for cytokine production, chemokine recognition and lymphocyte migration (Actor et al. 2009).

Cronobacter sakazakii is now considered an opportunistic pathogen that has been implicated in bacteremia, necrotizing enterocolitis (NEC), and neonatal meningitis (Healy et al. 2010). Although *C. sakazakii* infections are more common in neonates and premature babies, it has been reported that immune compromised adults are also susceptible to infections caused by this bacterium (Healy et al. 2010). The infant mortality rate associated with *C. sakazakii* infections ranges from 40 to 80% (Bowen et al. 2006). In addition, survivors often develop serious long-term neurological complications (Lai 2001). Despite the relatively low incidence of disease, the severity of these diseases has led to considerable interest in developing strategies for preventing or mitigating

infections. Therefore, the main goal of this research was to assess the ability of lactoferrin to inhibit adherence of *C. sakazakii* to a human cell line.

Materials and Methods

Organisms and growth conditions. Strains of *C. sakazakii* used in this study were obtained from K. Venkitanarayanan (Department of Animal Science, University of Connecticut) and conditions for their growth have been previously described (Quintero et al. 2011). Briefly, frozen stock cultures of each organism were thawed, plated onto Tryptic Soy Agar (TSA; Difco) and grown overnight at 37°C. A single colony was inoculated into 10 ml of Tryptic Soy Broth (TSB; Difco) and incubated aerobically, without shaking. Preliminary experiments revealed that adherence rates were highest when cells were in late log phase. Therefore, a 1% inoculum was incubated for 4 hours at 37°C without shaking. Cultures were harvested by centrifugation (3,184 x *g* for 8 minutes), washed once with phosphate-buffered saline (PBS) and re-suspended in minimal essential medium (MEM; Hyclone, Logan, Utah), supplemented with 10% fetal bovine serum (FBS; Hyclone). Minimal essential medium was pre-equilibrated at tissue culture conditions (5% CO₂, 95% relative humidity, 37°C). Based on additional preliminary experiments, strain 4603 repeatedly gave the high adherence rates on HEp-2 cells and was used for all subsequent experiments.

Lactoferrin. Two different types of lactoferrin (003 and 004) were obtained from Mead Johnson Nutrition (Evansville, IN). The lactoferrin was prepared as

concentrated stock solutions with distilled sterile water at a final concentration of 100 mg/ml. The solutions were filter sterilized using 0.22 µm filters.

Tissue culture cells. HEp-2 cells were obtained from the American Type Culture Collection (ATCC; Manassas, Virginia). Cells were grown in 75 cm² tissue culture flasks (Corning) containing 25 ml of MEM supplemented with 10% FBS in a CO₂ incubator at tissue culture conditions. Confluent Hep-2 cells were harvested by adding 0.5 ml of 0.25% Trypsin-EDTA Solution (Sigma) and incubating for 15 minutes at tissue culture conditions. Trypsin was inactivated with 0.5 ml of FBS. Cells were then seeded onto 12-mm diameter glass coverslips in 24-well tissue culture plates (Falcon) at approximately 3.6×10^5 viable cells per well, and 500 µl of MEM supplemented with 10% FBS was added to each well. Plates were incubated under tissue culture conditions 30 hours prior to the start of each experiment, or until confluency was reached.

Adherence Assays. Cell suspensions of *C. sakazakii* 4603 for HEp-2 cells were prepared as described above. Lactoferrin was mixed with bacterial cultures (in MEM supplemented with FBS) prior to addition to the tissue culture cells. The plates were incubated for 30 minutes at tissue culture conditions. Preliminary experiments indicated that 30 minutes of incubation were optimal for adherence of the bacterial strains to the HEp-2 tissue culture.

Quantitative Real Time PCR (qRT-PCR). Bacterial cells from adherence assays were detached by the addition of 1 ml of 0.1% Triton X-100 for 30 minutes at room temperature. Bacterial cells were harvested and centrifuged for

5 min at 10,000 x g, supernatant was discarded and pellet was resuspended in 180 µl of buffer ATL from the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Instructions provided in the manufacturer's manual for DNA extraction of Gram negative bacteria were followed to complete the extraction.

Quantitative real time PCR (qRT-PCR) was performed using a Mastercycler Realplex2 (Eppendorf AG, Hamburg, Germany). Each PCR was done in a 25 µl volume. The reaction mixture comprised 11.25 µl of the 20x SYBR solution and 2.5-µl Real-MasterMix (5Prime), 0.5 µM of each primer, and 1µl of DNA template (Martínez et al. 2009). Specific primers and amplification program were followed for each organism. (Table 1)

Results

Lactoferrin 003 inhibits adherence of *C. sakazakii* to HEp-2 cells. Adherence of *C. sakazakii* 4603 in the presence and absence of lactoferrin 003 was measured by qRT-PCR. Initial doses ranged from 0.1 to 1.0 mg/ml. However, no inhibition was observed at these concentrations (data not shown). Subsequently, doses of 2 to 50 mg/ml were tested. Adherence inhibition was observed at a minimum concentration of 10 mg/ml. Higher concentrations, however, did not show a further antiadhesive effect (Figure 1).

Lactoferrin 004 inhibits adherence of *C. sakazakii* to HEp-2 cells. Adherence of *C. sakazakii* 4603 in the presence and absence of lactoferrin 004 was performed as for lactoferrin 003. As before, adherence inhibition was observed

at a minimum concentration of 10 mg/ml and higher concentrations had no additional effect (Figure 2).

Discussion

Among the biological functions attributed to lactoferrin are antimicrobial, anti-inflammatory, and immunomodulatory activities (Kruzel et al. 2002; Legrand et al. 2008; Actor et al. 2009; Brock 2002; Ward et al. 2002). The mechanisms that account for the antimicrobial properties have been reported to be iron dependent and independent (Bullen et al. 1972; Arnold et al. 1980; Orsi 2004; Dalmastrri et al. 1988; Bortner et al. 1989); the latter implying direct interaction of lactoferrin with the bacterial cell surface (Appelmelk et al. 1994; Brandenburg et al. 2001; Shahriar et al. 2006). This direct interaction can result in the inability of the bacterium to adhere to host cell surfaces (Qiu 1998; Jenssen et al. 2009; Ochoa et al. 2003; DeVinney et al. 1999; Goosney et al. 1999). Previous studies have shown that lactoferrin prevents colonization of *Haemophilus influenzae*, degrades virulence proteins secreted by *Shigella flexeneri*, and inhibits adherence of *Salmonella typhimurium*, as well as it interferes with the Type III Secretory System present in many pathogenic bacteria (Qiu 1998; Jenssen et al. 2009; Gomez et al. 2003; DeVinney et al. 1999; Goosney et al. 1999; Gomez et al. 2001; Ochoa et al. 2003; Bessler et al. 2006).

Our results show that two different types of lactoferrin inhibit adherence of *Cronobacter sakazakii* 4603. The mechanism by which *C. sakazakii* adheres to host cell surfaces has not been well studied, although it has been established

that an outer membrane protein A (OmpA) might play an important role in the attachment and invasion of Caco-2 cells (Kim et al. 2008). The mechanism by which lactoferrin inhibits adherence of *C. sakazakii* to HEp-2 cells requires further investigation to determine whether it is strictly dependent on direct interaction of lactoferrin with the bacterial cell surface. Interestingly, lactoferrin inhibits *C. sakazakii* 4603 at a minimum required concentration of 10 mg/ml, although inhibition does not increase significantly by further increasing the lactoferrin dose. This suggests that lactoferrin may directly interact with the bacterial surface of *Cronobacter sakazakii*, as a mechanism to inhibit its adherence to epithelial cells. Therefore, upon saturation, there will not be a greater antiadherent effect with higher lactoferrin concentrations.

Conclusion

Altogether, our results show that lactoferrin 003 and lactoferrin 004 inhibit adherence of *Cronobacter sakazakii* 4603 to HEp-2 cells. Although the minimum concentration to inhibit adherence was found to be 10 mg/ml, further research is needed to establish the means by which lactoferrin interacts with *C. sakazakii* to inhibit its adherence.

The first, and a crucial step in bacterial pathogenesis, is bacterial adhesion to host cell surfaces. Agents that inhibit pathogen adherence, such as lactoferrin, may be considered as food ingredients that might serve as a prophylactic treatment to prevent infections.

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Figure 1. Adherence of *Cronobacter sakazakii* 4603 to HEp-2 cells in the presence of Lactoferrin 003 at doses ranging from 0 to 50 mg/ml. Statistically significant effects are indicated by an asterisk.

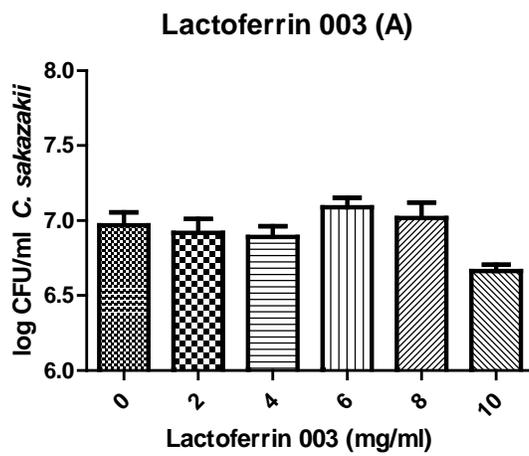
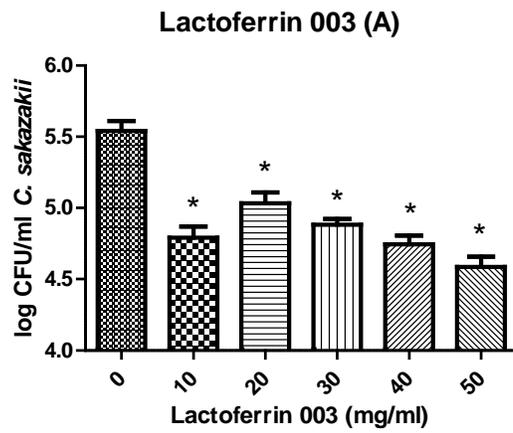
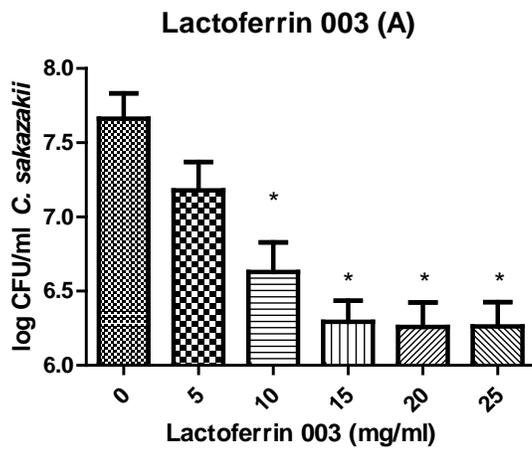


Figure 2. Adherence of *Cronobacter sakazakii* 4603 to HEp-2 cells in the presence of Lactoferrin 004 at doses ranging from 0 to 50 mg/ml. Statistically significant effects are indicated by an asterisk.

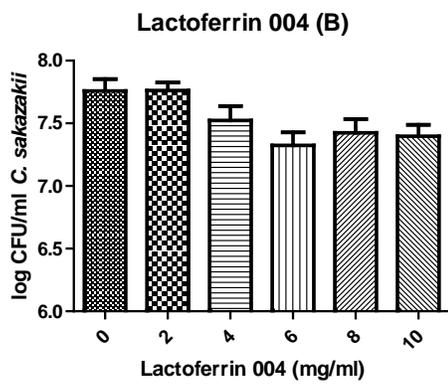
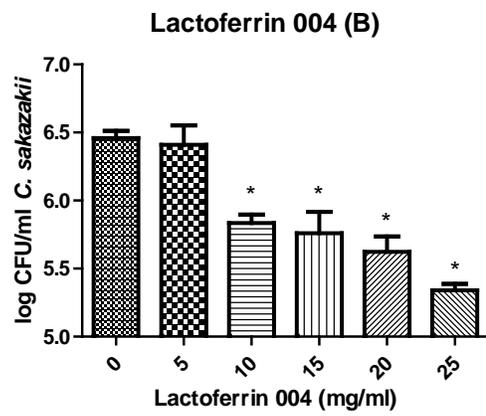
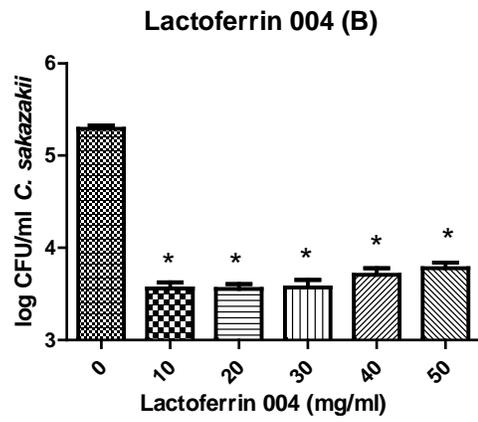


Table 1. Primers and qRT-PCR program

Table 1.

Organism	Primers (5' - 3')	Amplification Program
<i>C. sakazakii</i>	For TATAGGTTGTCTGCGAAAGCG Rev GTCTTCGTGTGCGAGTTTG	denaturation at 95°C for 10 s, 45 cycles consisted of denaturation at 95°C for 5 s, 62°C for 20 s for annealing and extension.

Chapter 5

Conclusion

In this research we established that several food-grade prebiotic carbohydrates, plant extracts, and other naturally-derived molecules inhibit adherence of enteric pathogens to epithelial cells. Specifically, GOS and PDX, alone and in combination, as well as lactoferrin inhibit adherence of *Cronobacter sakazakii* to a HEP-2 cell line. Furthermore, it was determined that a high molecular weight component from cranberry, mannan from yeast, and chitin oligosaccharides, inhibit adherence of EPEC, EHEC, and *Salmonella typhimurium* to epithelial cells. The major findings of this research are described below.

- GOS and a blend of GOS-PDX effectively reduce adherence of *C. sakazakii* in tissue culture experiments.
- There is enough similarity between GOS and human milk oligosaccharides to be effective in inhibiting adherence at the concentration used in this study.
- The antiadherent effect of prebiotic oligosaccharides varies among different strains and epithelial cell lines.
- Different oligosaccharides and plant extracts inhibit adherence of EPEC, EHEC, and *Salmonella typhimurium* to tissue culture cells.
- The high molecular weight component of cranberry inhibits adherence of the three enteric pathogens used in this study, at concentrations ranging from 0.2 to 2 mg/ml.
- Mannan from yeast cells inhibits adherence of EPEC and *Salmonella typhimurium* at a minimum concentration of 75 and 10 mg/ml, respectively.

- Mannan from konjac root does not inhibit adherence of any of the tested enteric pathogens.
- Different fractions of chitin oligosaccharides reduce adherence of EPEC to a HEp-2 cell line. Reduction in adherence is mainly due to adherence inhibition rather than by a bactericidal effect.
- Lactoferrin inhibits adherence of *Cronobacter sakazakii* to a human epithelial cell line,
- The minimum lactoferrin concentration required for *C. sakazakii* adherence inhibition is 10 mg/ml; higher concentrations do not exhibit higher adherence inhibition.
- Collectively, these results provide a basis for development of natural prophylactic agents that may be effective at reducing or preventing infections by enteric pathogens.
- In addition, the results of this research suggests that food-grade anti-adherent agents may serve as a substitute for the widespread use of antibiotics whose use in animal agriculture may soon be restricted.